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# THE EFFECTS OF THIOUREA AND PHENYLTHIOUREA UPON THE DEVELOPMENT OF ELEUTHERODACTYLUS RICORDII

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The leptodactylid toads of the genus *Eleutherodactylus* are unusual among anurans in that they lay large-yolked, unpigmented, terrestrial eggs and have no aquatic larval stage. The embryology of one member of this group, the Jamaican species *E. nubicola*, has been described in some detail (Lynn, 1942) and it was pointed out that during the development within the egg membranes certain characters which are found in ordinary aquatic tadpoles appear very transiently while others are never present at all. Thus, for example, the embryo possesses a broad vascular tail which is large and well developed during the latter part of intra-oval life but degenerates and disappears near the time of hatching. Neither external nor internal gills are ever formed, there is no true opercular cavity and the ventral sucker and horny teeth of the typical tadpole are entirely lacking. The fore and hind limbs appear simultaneously and grow steadily throughout the embryonic period and at the time of hatching the young emerge as fully developed little frogs.

It is well known that in ordinary anurans such as *Rana pipiens* the metamorphosis of the tadpole into the frog is brought about through the agency of the thyroid gland which, late in larval life, begins a period of intense secretory activity resulting in a heightened level of thyroid hormone in the blood stream. The loss of gills and tail, shortening of the intestine, rapid growth and differentiation of the limbs, shedding of the chitinous beak, appearance of the tympanum and many other features of metamorphosis have been shown to be directly dependent upon this gradually increasing level of thyroid hormone. Thyroidectomized tadpoles remain in the larval state indefinitely unless thyroxin or iodine is administered to them. On the other hand, if normal tadpoles are treated with thyroxin at an early age they can be made to metamorphose precociously, producing tiny, but perfectly formed young frogs.

In view of these facts it seems possible that the telescoping of the larval stages in the development of *Eleutherodactylus* might be regarded as an exaggeratedly precocious metamorphosis resulting from an unusually early and intense functioning of the thyroid in this genus. The author has earlier presented some evidence, based on the histology of the thyroid at different embryonic stages, indicating that the gland is indeed precociously activated in this animal (Lynn, 1936). More recently Brink (1939) has made a study of the histology and cytology of the thyroid in *Arthroleptella bicolor villiersi*, a South African ranid with a somewhat similarly abbreviated larval history, and has concluded that in this form also, the release of the thyroid hormone into the blood stream occurs at an unusually early stage.

It is clear that this evidence based upon the histological picture presented by the thyroid must be supplemented by experimental evidence before it can be regarded as conclusive. In the summer of 1941 the present author, working in Jamaica, B. W. I., attempted an experimental approach by removal of the pituitary anlage in the early embryo of *E. nubicola.* This operation, since it results in absence of the thyrotrophic hormone of the pituitary, prevents normal differentiation and functioning of the thyroid gland and thus provides a method for ascertaining the normal role of the thyroid in the developmental processes under investigation. Unfortunately these experiments were inconclusive because of the difficulty encountered in keeping the animals alive after the operation. Although the operation was relatively simple and involved removal of only a small bit of tissue, the embryos did not heal with the readiness exhibited by most amphibian larvae and despite repeated attempts, no operated animals survivied more than a few days.

With the recent development of various thyroid-inhibiting drugs, thiourea and related compounds (Kennedy, 1942; Richter and Clisby, 1942) and a number of the sulfonamides (Mackenzie, Mackenzie and McCollum, 1941; Mackenzie and Mackenzie, 1942, 1943; Astwood, 1943; Astwood, Bissell and Hughes, 1945), a new approach to the problem is possible since administration of these drugs will effectively block the production of thyroid hormone without the necessity of any surgical treatment.

The present paper is a report of such experiments carried out on the embryos of *Eleutherodactylus ricordii planirostris* (Cope), a species which is native to Cuba but has now, through accidental introductions, become well established in many parts of Florida.

## Materials and Methods

The eggs used for this study were collected at Gainesville, Florida between August 19 and September 2, 1946.[1] The breeding habits of *E. ricordii* in Florida have been described by Deckert (1921), Skermer (1939) and Carr (1940). The eggs are laid in moist situations under loose boards or stones and the female remains with the eggs until they hatch. Goin (1947), in a study made at Gainesville, found that the average number of eggs per clutch is 16 and that the average period of development within the egg membranes is 15.6 days. For the present work 15 clutches of eggs were used. Each clutch was designated with a letter, the 15 groups thus constituting Series A to O. The total number of eggs available was 193 and the number per clutch ranged from 5 to 21.

Although these eggs are normally terrestrial, experience with the Jamaican species had shown that they can survive when immersed in water. It was therefore possible to administer the drugs simply by raising the eggs in the solutions, thus avoiding the necessity of injection. In each experiment a few eggs were kept on moist sand in a small flower-pot covered with a glass plate in order to simulate natural conditions; others were raised in tap-water and still others were raised in tap-water containing the appropriate concentration of the drug to be tested.

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[1] The author is indebted to Prof. J. Speed Rogers, Head of the Department of Biology, The University of Florida, for laboratory facilities during the course of the experiments and to Dr. C. J. Goin for suggestions and assistance in the collecting of specimens. Sectioning and study of the material was carried out at The Catholic University of America and at The Marine Biological Laboratory, Woods Hole, Massachusetts.

All of the eggs in a single batch are at the same stage of development at any given time but, of course, those of different batches were at various stages when collected. For this study only those series were used which were collected at the neural plate stage or earlier. All eggs were kept in the laboratory until they reached the early limb-bud stage before any treatment was instituted. This procedure was adopted because it insured that all embryos received treatment for comparable periods and also because previous experience had shown that successful removal of the jelly coats and vitelline membrane is difficult at earlier stages. At the limb-bud stage the thyroid gland is not yet differentiated (Lynn, 1936, 1942) so that in all of these experiments the treatment with thyroid-inhibiting drugs was instituted before the beginning of thyroid function.

Removal of the egg membranes was carried out in sterile Holtfreter's solution by means of finely ground watchmaker's forceps.

Daily observations were made under the binocular microscope and at various intervals embryos were fixed for sectioning. Fixation was in a 1:1 mixture of Bouin's fluid and cellosolve. The embryos were later dehydrated in cellosolve, cleared in xylol, sectioned at 10 $\mu$ and stained with Mallory's triple stain.

## RESULTS

1. Effect of the egg membranes upon the developmental rate of eggs raised in water.

Although eggs placed directly into tap-water were found to survive and to develop into froglets of normal appearance it became obvious early in the course of the work that such eggs were markedly retarded in their rate of development as compared with those kept in air on moist sand. On the other hand, if the jelly layers and vitelline membranes were removed from the eggs to be kept in fluid, then the developmental rate closely paralleled that of eggs kept on sand with all their coverings intact.

The details of a single experiment may be cited to illustrate this effect. The eggs of Series D were in early cleavage when collected on August 19. They were kept on moist sand for 5 days at which time all were well-developed embryos with both pairs of limbs present as buds, large vascular tails and lightly pigmented bodies (the "limb-bud stage"). At this time some of the eggs were dissected free of the surrounding jelly and membranes and placed in tap-water, others were placed in tap-water with all coverings intact and others were kept on moist sand. The eggs kept on sand developed normally and all hatched 10 days later (15th day of development). Eggs in tap-water without membranes paralleled those kept on sand. Differentiation and growth of limbs and digits, intensification of pigment, growth and later degeneration of the tail and other grossly visible changes occurred concomitantly in the two groups and at the time when the eggs kept in air were hatching, those kept in water were indistinguishable from them. Young frogs which were left in water after this time died within two days but those removed to moist sand lived normally. This is undoubtedly to be attributed to the change from cutaneous to pulmonary respiration which occurs at this time. During embryonic existence in the absence of any gills the respiration is cutaneous, probably mainly through the thin-walled vascular tail. At late stages the tail begins to degenerate and it usually disappears within a day after hatching, when the lungs have come into function.

The eggs which were left in their membranes and raised in water showed a retardation almost immediately. Differentiation of the limbs was slow and the difference in pigmentation between these animals and the members of the control groups was particularly noticeable. By the 15th day of development, the time when the controls hatched, these embryos were at the stage which the controls had reached on the 8th day. They continued to develop slowly and reached what appeared to correspond to the hatching stage on about the 26th day. The embryos seemed weak however and none hatched spontaneously. When freed from the egg membranes by forceps they swam sluggishly, but when removed to moist sand they survived successfully.

The retarding effect of the egg membranes is shown in Figures 1 and 2 which are photographs of two individuals of Series H. These embryos were eleven days old when photographed and both had been kept in tap water from the 5th day of development. The embryo shown in Figure 2 had all coverings intact while that shown in Figure 1 had the membranes removed at the time of its immersion in water. The difference in the differentiation of the limbs and digits is particularly striking but sectioned material reveals that this is merely one aspect of a general retardation in developmental rate. It seems probable that this effect is due to a reduction of the rate of gas diffusion to and from the embryo, but no determinations of respiratory rate have been made to test this.

This effect having been demonstrated, all the experiments with thyroid-inhibiting drugs were carried out with eggs freed from the jelly layers and vitelline membrane.

2. Effects of thiourea treatment upon development.

A total of 71 eggs taken from 12 different batches was used for study of the effects of thiourea treatment. Three different concentrations were tested and, as previously noted, separate controls were run for each batch of eggs.

The lowest concentration of thiourea used was 0.001 per cent. Only 8 eggs, taken from Series A and B, were exposed to this concentration. The development of these did not seem to be affected in any way, the rates of growth and differentiation being the same as those of tap-water controls, and use of this concentration was therefore discontinued.

Twenty-four embryos from 5 different series of eggs were raised in 0.005 per cent thiourea. These animals showed no significant retardation in their rate of devel-

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PLATE I

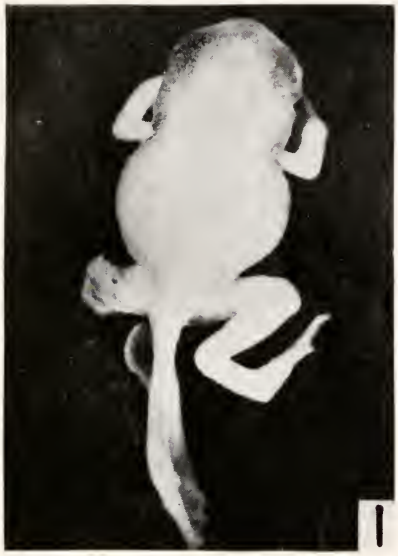
FIGURE 1. Specimen from Series H, removed from jelly layers and vitelline membrane and placed in tap-water on the fifth day of development. Preserved and photographed on the eleventh day. × 8.

FIGURE 2. Specimen from Series H, raised in tap-water from the fifth to eleventh days with jelly layers and vitelline membrane intact. (Coverings removed before photographing.) × 8.

FIGURE 3. Specimen from Series H, raised in 0.005 per cent thiourea from the fifth to eleventh day of development. × 8.

FIGURE 4. Specimen from Series H, raised in 0.005 per cent phenylthiourea from the fifth to eleventh day of development. × 8.

PLATE I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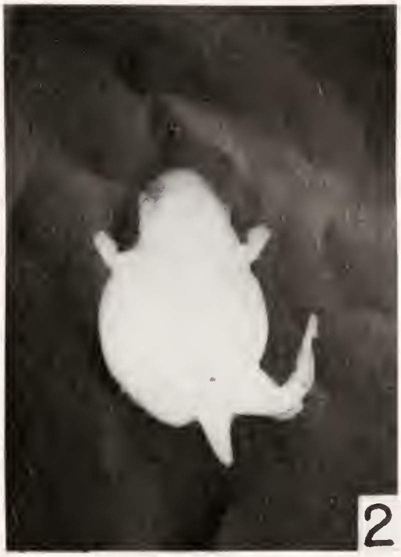
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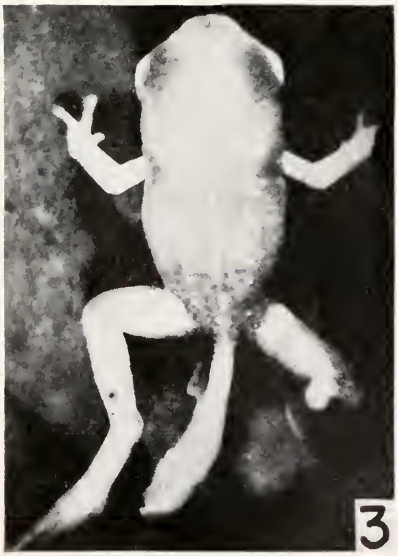
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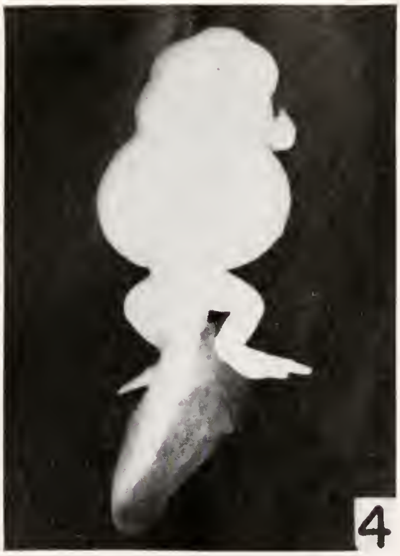
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opment but at the time when the tap-water controls were exhibiting marked degeneration of the tail, the tails of the experimental frogs remained large and vascular. This being the case, the latter animals were able to survive in water for an indefinite period while controls left in water invariably died when the tails had been reduced to small size. Because of the author's short stay in Florida, the longest period of survival of these experimental larvae beyond the time of death of the controls was 9 days. However, these animals were all active and healthy in appearance when fixed and there is no reason to suppose that they could not have lived much longer if some means of feeding them could have been found.

Figures 3 and 6 illustrate the effect of treatment with 0.005 per cent thiourea. The animal shown in Figure 3 is a specimen from Series H photographed at 11 days of age after 6 days in this solution. Comparison with the tap-water control of the same series (Fig. 1) shows that there is no significant difference between the two at this time ("pre-hatching stage"). Figure 6 shows an individual of Series N which was kept in 0.005 per cent thiourea for 11 days and a tap-water control of the same series is shown in Figure 5. It will be seen that the latter has passed the "hatching stage," having lost the tail and assumed the adult body form, while the experimental animal shows no signs of tail degeneration. Differentiation of the limbs in the two is, however, essentially the same.

Thirty-nine embryos from 7 different series of eggs were raised in 0.05 per cent thiourea solution. This concentration caused a definite retardation of development which first became apparent about 4 or 5 days after the beginning of treatment. From this time on, the experimental animals lagged behind the tap-water controls so that when the latter reached the "hatching stage," the former still had poorly developed digits, large vascular tails and ill-defined pigment patterns. Further development of these embryos was extremely slow and animals kept in 0.05 per cent thiourea for 10 days beyond the "hatching time" of the controls still exhibited several embryonic features in addition to the large larval tail. This is shown in Figures 7 and 8. These two embryos of Series C had been in fluid for 10 days. The control (Fig. 7) is very near the hatching stage with a much reduced tail and well-developed digits. The experimental animal (Fig. 8) has a tail of maximum size and shows considerable retardation of limb differentiation and pigment pattern development.

It is unfortunate that considerations of time made it impossible to carry these animals for longer periods beyond the "hatching time" for it appears probable that, in the case of embryos treated with 0.05 per cent thiourea, certain of the developmental features are not merely retarded but actually inhibted. Complete differentiation of the limbs was never attained and there was never any sign of metamorphic

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PLATE II

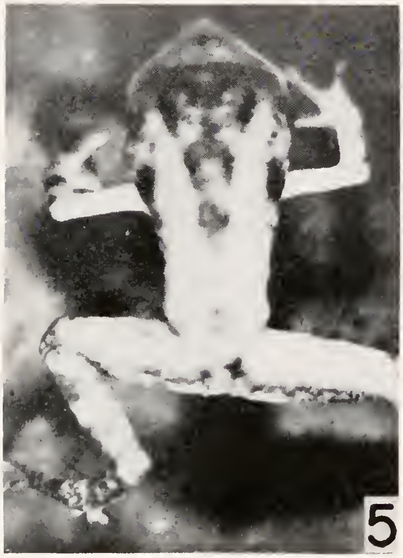
FIGURE 5. Specimen from Series N, raised in tap-water from the fifth to the sixteenth day of development. × 8.

FIGURE 6. Specimen from Series N, raised in 0.005 per cent thiourea from the fifth to the sixteenth day of development. × 8.

FIGURE 7. Specimen from Series C, raised in tap-water from the fifth to the fifteenth day of development. × 8.

FIGURE 8. Specimen from Series C, raised in 0.05 per cent thiourea from the fifth to the fifteenth day of development. × 8.

PLATE II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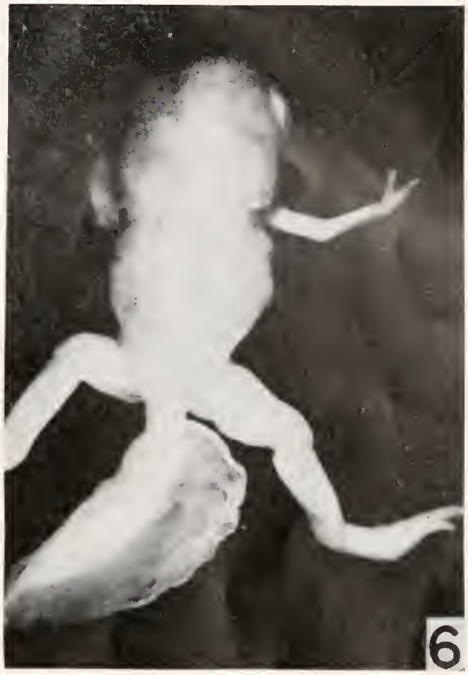
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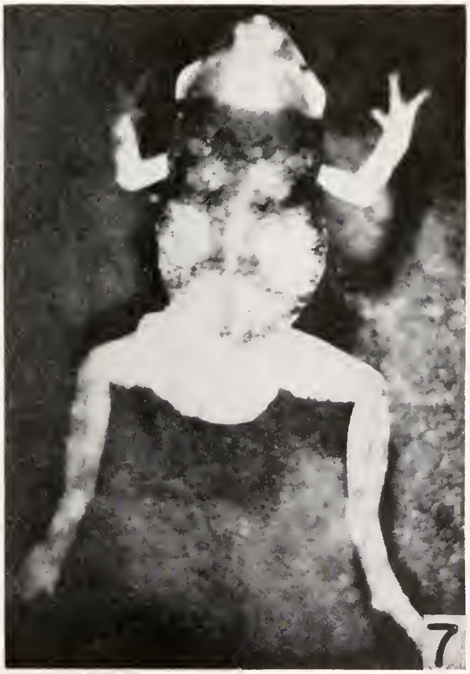
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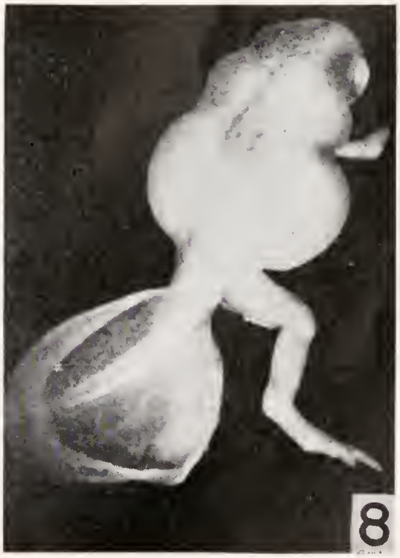
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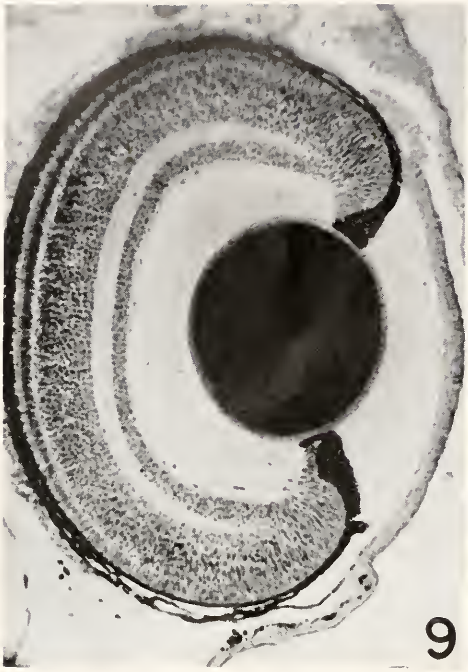
## PLATE III



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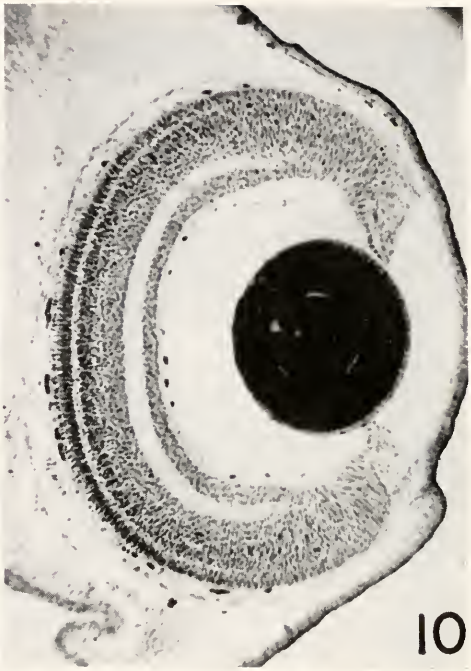

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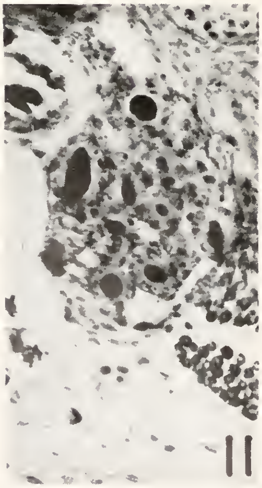

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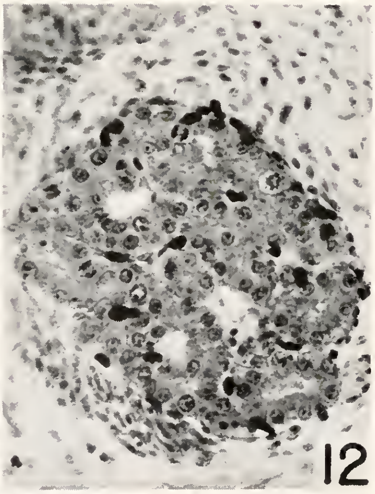

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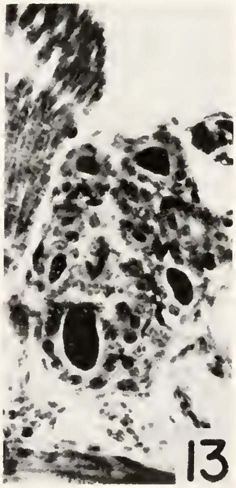

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degeneration of the tail during the course of the experiment. These are clearly effects which would be expected to result from inhibition of the metamorphosis-inducing properties of the thyroid but, since the animals when fixed still retained considerable amounts of yolk, the possibility remains that the effect is attributable to a general retardation of metabolic rate. Raising the embryos in 0.05 per cent thiourea for much longer periods, until complete utilization of the yolk, would doubtless settle this point.

3. Effects of phenylthiourea treatment upon development.

Twenty eggs from 5 different series were treated with 0.005 per cent phenylthiourea. These all showed a retardation of development which was essentially the same as that caused by 0.05 per cent thiourea. Degeneration of the tail was prevented and complete differentiation of the limbs and digits was never attained.

An additional and very striking effect produced by phenylthiourea, however, was a rapid and complete loss of pigment. At the limb-bud stage, when treatment was instituted, the embryos had a light peppering of melanophores over the dorsal surface and the pigmented coat of the retina was quite black. In every case, however, the experimental larvae were noticeably lighter than the controls by the third day after the beginning of treatment, and by the fifth or sixth day all visible dark pigment had disappeared. The eyes became white and the skin took on a translucent golden appearance indicating decoloration of the melanophores but not of the lipophores. It was also noted that these embryos after about ten days in the solution gave evidence of an abnormally high blood pressure or increased strength of heart-beat, the head and fore-limbs moving rhythmically with each heart-beat. The heart rate was not significantly different from that of the controls however.

An illustration of the effect of 0.005 per cent phenylthiourea upon general development and pigmentation will be seen in Figure 4. The animal shown in this photograph is another individual of Series H which was kept in the phenylthiourea solution for six days after the limb-bud stage. Comparison with the tap-water control (Fig. 1) or the animal kept in 0.005 per cent thiourea (Fig. 3) shows the pronounced depigmentation and the decided retardation in differentiation of the limbs. The loss of pigment in the pigmented coat of the eye is best seen in sectioned material as shown in Figures 9 and 10 which are photographs of sections of the eyes of the same animals shown in Figures 1 and 4. The pigmented coat and iris of the phenylthiourea-treated animal (Fig. 10) is almost completely decolorized despite the fact that this animal had been exposed to the drug for only 6 days.

4. Effects of thiourea and phenylthiourea upon the histology of the thyroid gland.

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PLATE III

FIGURE 9. Section through the eye of the control animal shown in Figure 1. × 150.

FIGURE 10. Section through the eye of the phenylthiourea-treated animal shown in Figure 4. × 150.

FIGURE 11. Central section through the thyroid gland of the control animal shown in Figure 1. × 300.

FIGURE 12. Central section through the thyroid gland of the phenylthiourea-treated animal shown in Figure 4. × 300.

FIGURE 13. Central section through the thyroid gland of the thiourea-treated animal shown in Figure 3. × 300.

Examination of serial sections of control animals and those subjected to the various treatments described above provides a close correlation between the grossly visible effects upon development and the histological changes induced in the thyroid.

The results of treatment with 0.005 per cent phenylthiourea may conveniently be described first. Sections of the thyroids of experimental and control embryos fixed on the third day of treatment (eighth day of development) already exhibit well-marked differences. The control thyroid consists of relatively few small, primary follicles with low cuboidal epithelium and with the lumina occupied by a homogeneous blue-staining colloid. It represents a fairly early stage and only a mild degree of thyroid activity. The glands of the treated animal are only slightly enlarged but the follicular epithelium is predominantly columnar, some vacuolation of the colloid has occurred and the vascularization of the thyroid has increased. By the sixth day of treatment the contrast is much more striking and this stage has been chosen for illustration. Figure 11 is a photomicrograph of a central section of the thyroid of a tap-water control from Series H at this time. It will be noted that the follicular epithelium is cuboidal to low columnar and that all follicles contain fairly large masses of homogeneous red-staining colloid. This is a relatively active, but not a hyperactive, gland. The thyroid of the treated embryo is shown in Figure 12. It is markedly enlarged and the follicular epithelium is hyperplasic. Mitotic figures are common, three of them being seen in this photograph. Most follicles are completely collapsed and those which are not contain almost no stainable colloid. The hyperemia is indicated by the numerous blood corpuscles scattered about between the follicles. Essentially this same picture of intense activity is seen in all later stages studied which include animals up to the twenty-fourth day of development (nineteenth day of treatment) and the later stages are therefore not illustrated. There is no evidence in this material of any regression in activity during the period studied but perhaps this would have been observed if treatment could have been continued for a longer time.

The thyroids of animals treated with 0.05 per cent thiourea present the same picture as that produced by 0.005 per cent phenylthiourea and therefore need not be discussed in detail. It will be remembered that both of these treatments caused the same retardation in development.

Thyroids of animals kept in 0.005 per cent thiourea differ from those of controls only in that they are slightly enlarged and show increased vascularity. There is no significant difference in the amount or nature of the colloid present or in the height of the follicular epithelium. The thyroid shown in Figure 13 is that of the animal shown in Figure 3 which had been in 0.005 per cent thiourea for 6 days. It may be compared with the control of the same age (Figure 11). Animals treated for longer periods show no more pronounced effects. Despite the slight histological change exhibited by the thyroid in this case, some change in the amount or nature of the hormone produced must be postulated since tail degeneration is definitely prevented by this concentration.

The available sectioned material of animals treated with 0.001 per cent thiourea shows no points of difference from the controls. This was to have been expected since this concentration produced no detectible effects on development.

## Discussion

Although only a few studies of the effects of thyroid-inhibiting drugs upon amphibians have as yet been made it has been conclusively demonstrated that these substances can produce effects upon the larva which are comparable to those resulting from thyroidectomy. Gordon, Goldsmith and Charipper (1943, 1945) showed that *Rana pipiens* tadpoles kept in 0.033 per cent thiourea retain the larval tail, gills and mouth-parts and fail to attain complete differentiation of the limbs. In other words they do not metamorphose, although they do continue to grow and may reach excessive sizes. Metamorphosis usually occurs promply when treatment ceases, although it may be delayed if treatment has been of long duration. Similar effects produced by thiouracil in a concentration of 1:2000 have been reported for *Rana clamitans* (Hughes and Astwood, 1944) and for *Rana pipiens* (Lynn and Sister Alfred de Marie, 1946).

On the basis of the present work it is clear that in *Eleutherodactylus,* as in *Rana,* there are certain developmental features which are dependent upon thyroid stimulation and are inhibited when normal production of the thyroid hormone is interfered with. The most noteworthy of these features are the resorption of the larval tail and the completion of differentiation of the limbs and digits. On the other hand, however, these experiments show that many of the developmental processes which are under thyroid control in ordinary anurans are to a greater or less degree, independent of such control in *Eleutherodactylus.* Thus even under conditions of what seems to be complete thyroid inhibition (treatment with 0.05 per cent thiourea or 0.005 per cent phenylthiourea) no tadpole-like mouth-parts, operculum or gills are ever formed and the limbs do develop to a considerable degree before showing any inhibition. In other words the treatment with thyroid-inhibitors does not result in the appearance of any larval characters which are normally absent in the species but it does cause an indefinitely prolonged retention of certain features which are normally very transient.

In an earlier discussion of this matter (Lynn, 1936) the author has pointed out that the evolutionary change which resulted in the atypical life history of *Eleutherodactylus* could conceivably have been brought about through a relatively simple genetic change, namely one which resulted in a precocious activation and functioning of the endocrine complex governing metamorphosis. The telescoping of the larval stages and early assumption of the adult body form could result from this and the later stages of intra-oval development could then be properly regarded as a precocious metamorphosis. The present experiments indicate quite clearly that the evolution of terrestrial development in these frogs can not be reduced to such simple terms. The thyroid stimulus undoubtedly plays a part in some of the later differentiations but many features of the embryogeny are carried out independently of the thyroid and it is obvious that in this anuran the genetic constitution is such that many of the tissues are able to undergo complete differentiation to the adult form without the endocrine intervention which is so essential in most amphibians. The development of the *Eleutherodactylus* embryo can, therefore, be more accurately described as a "direct development" rather than a "prococious metamorphosis within the egg."

The depigmentation effect exhibited by phenylthiourea but not by thiourea merits some discussion. Even before the discovery of the goiterogenic properites of phenylthiourea it was reported by Richter and Clisby (1941) that continued administration of this substance to black rats causes graying of the hair, and also that cessation of treatment is followed by return of pigment. This phenomenon has been further studied by Dieke (1947) who finds also that alpha-naphthyl thiourea causes depigmentation of the skin of the rat. Neither of these drugs, in the doses used, had any effect upon the eye pigment of the rat.

Juhn (1944, 1946) has reported an effect of thiouracil administration upon feather pigmentation in the Brown Leghorn fowl but this seems to be attributable to the thyroid inhibition rather than to any direct influence upon the pigment cells for the modifications produced are the same as those which result from thyroidectomy.

Only one published account is concerned with the effects of thiourea derivatives upon the pigmentation of amphibians. This report (Lynn and Sister Alfred de Marie, 1946) records a reversible blanching observed in tadpoles of *Rana pipiens* raised in 0.05 per cent thiouracil. Further experiments in this laboratory have revealed that a definite depigmentation of the skin of *Rana pipiens* tadpoles is also produced by treatment with allylthiourea, phenylthiourea, amenobenzoic acid and sulfanilamide. In none of these cases, however, was the pigmentation of the eyes lost. The *Eleutherodactylus* embryo thus seems to be particularly susceptible to this action, showing much more complete and rapid depigmentation than does *Rana pipiens.*

It is noteworthy that a depigmentation of the skin and eyes very similar to that caused by phenylthiourea was reported much earlier (Lewis, 1932) in *Rana sylvatica* larvae treated with certain of the indophenol dyes. Moreover Figge (1938a), testing the effectiveness of these dyes upon various amphibian larvae, found a marked difference in sensitivity in different species. Larvae of *Necturus* were very readily depigmented in relatively low concentrations of the dyes; larvae of *Rana sylvatica* were somewhat less sensitive; larvae of *Amblystoma mexicanum* and *Rana catesbiana* were still less readily affected and *Amblystoma punctatum* larvae were least sensitive of all. Figge points out that this order of sensitivity parallels the order of metabolic rate of the different animals studied. *Necturus,* the most sensitive, has the lowest metabolic rate; *A. punctatum,* the least sensitive, has the highest; while *A. mexicanum* is intermediate in both respects. It is unfortunate that, because of the lack of any studies upon the metabolism of the *Eleutherodactylus* embryo, no conclusions concerning a possible relation between metabolic rate and sensitivity to the depigmentation effects of phenylthiourea can be drawn.

The basis for the depigmentation effect of the indophenol dyes has been investigated by Figge (1938b, 1939, 1940, 1941) who finds that phenol indophenol does not destroy pigment granules once formed but does prevent further formation of granules by affecting the enzyme system responsible for pigment production. Specifically, it was found that the dye inhibits the enzyme tyrosinase and thus prevents the production of melanin by the action of tyrosinase on tyrosine. The mechanism of the inhibition is apparently to be found in the fact that the dye shifts the oxidation-reduction potential of the substrate away from the optimum potential for tyrosinase activity. Presumably any substance which would cause such a shift in substrate potential would be equally effective in inhibiting pigment formation.

In view of these findings for the indophenol dyes, it is not surprising that recent studies indicate that the depigmentation effect of phenylthiourea and others of the thiourea derivatives are also to be attributed to tyrosinase inhibition although whether the precise mechanism of inhibition is the same as that which seems to be obtained in the case of the dyes has not as yet been ascertained. Bernheim and Bernheim (1942) demonstrated inhibition of tyrosinase in vitro by phenylthiourea (phenylthiocarbamide), Paschkis, Cantarow, Hart and Rakoff (1944) have shown the effect for thiouracil, glutathione, cysteine, ascorbic acid, para-aminobenzoic acid, sodium sulfathiazole and sulfadiazine and Du Bois and Erway (1946) found that alpha-naphthyl thiourea is almost as effective as phenylthiourea in this respect while allyl-thiourea and thiourea are effective only in higher concentrations. The results of the present work are in agreement with this finding for, although 0.005 per cent phenylthiourea caused rapid and complete depigmentation of the embryo, thiourea, even at a concentration of 0.05 per cent, produced no discernible pigmentary changes.

It is of interest to note that in Figge's studies with the indophenol dyes these substances were found to produce, in addition to the pigmentary disturbances, specific effects on the eyes of some of the treated animals. The cells of the retina, lens and cornea were disoriented and the whole optic cup was collapsed and folded. No such effects have been observed in any of the animals subjected to the treatments employed in the present study. Even when the pigmented layer of the retina was completely lacking in melanin, the retinal layers themselves were normal in size and arrangement (Fig. 10). In this respect, therefore, the action of phenylthiourea differs from that of the indophenol dyes.

## SUMMARY

The leptodactylid toad, *Eleutherodactylus ricordii planirostris* (Cope), is unlike most anurans in that it possesses no aquatic larval stage. Its eggs are laid on land, beneath stones or logs. After about two weeks development within the egg, the young frogs hatch with the adult body form. It was found that eggs immersed in water will develop normally and at the usual rate providing the jelly layers and vitelline membranes are removed. In an attempt to ascertain to what degree the suppression of larval characters and the early assumption of the adult body form are dependent upon the activity of the thyroid gland, developing eggs were raised in solutions of thyroid-inhibiting drugs.

Embryos placed in 0.05 per cent thiourea or 0.005 per cent phenylthiourea on the fifth day of development failed to attain complete differentiation of the limbs and retained the larval tail, so that they were still embryonic in appearance 10 days after the tap-water controls had become complete little frogs. Animals raised in 0.005 per cent thiourea exhibited no retardation in limb development but did retain the larval tail so long as treatment was continued. Treatment with 0.001 per cent thiourea seemed to have no effect on development. Histological study of the thyroid glands of treated and control animals showed marked hyperplasia, hyperemia and reduction in colloid volume in the thyroids of specimens raised in 0.05 per cent thiourea or 0.005 per cent phenylthiourea. Thyroids of animals treated with 0.005 per cent thiourea showed slight hyperplasia and hyperemia but no significant differences in colloid volume as compared with controls. Thyroids of specimens raised in 0.001 per cent thiourea seemed to be unaffected. It appears that, in *Eleutherodactylus,* the loss of the larval tail and the complete differentiation of the limbs are features which are under thyroid control. On the other hand, the suppression or telescoping of many of the larval features cannot be attributed to thyroid activity since it occurs even under conditions of what seems to be extreme thyroid inhibition.

The embryos raised in 0.005 per cent phenylthiourea showed a rapid loss of pigment which involved not only the skin but also the pigmented coat of the eye. This is probably the result of the well-demonstrated inhibitory effect of this drug upon tyrosinase melanin formation.

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# CAFFEINE EFFECTS ON FERTILIZATION AND DEVELOPMENT IN ARBACIA PUNCTULATA

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The effects of caffeine as a dietary factor upon the reproductivity and growth of mammalian species have been reported for rabbits by Stieve (1931), and for rats by Cheney (1944) and Bachmann et al. (1946), without uniformity in conclusions. None of these studies described the fundamental action of caffeine directly upon the gametes per se nor upon the early stages of development.

The effects of so many chemical factors in the environment upon the fertilization and development of the Arbacia egg are so well known, the comparative literature is so abundant, and the egg itself so ideal for studies of the effect of a substance upon permeabilities, cleavage rates, and developmental symmetry, that the Arbacia egg was chosen to investigate the critical factors underlying the influence of caffeine. The effects of caffeine were tested upon the gametes, zygote, and early developmental stages.

In 1945, it was reported by the author that caffeine, in concentrations of M/1000 (0.02 per cent) or above, depresses the $O_2$ consumption of the zygote as demonstrated by the Warburg-Barcroft micro-respirometer technique. It is the purpose of the current investigation to determine the relative sensitivity of the gametes to caffeine, its effect upon the cleavage stages and subsequent developmental phases through the mature pluteus larva, and the degree to which different concentrations retard and inhibit development.

## METHODS AND MATERIALS

*Arbacia punctulata* gametes, shed directly into sea water (SW) and into eight different concentrations of caffeine-in-sea-water (CSW), after fifteen minutes were mixed for fertilization and allowed to develop to the following selected stages: fertilization membrane (FM), streak stage, 2-, 4-, 8-celled stage, late cleavage, blastula, gastrula, and pluteus larva. All eggs per experiment were obtained from the same female and all sperm from one male. Observations were made over a 72-hour period for the comparison of the developmental rate and form in SW and CSW. Normal time rates for the development of Arbacia were accepted as stated by E. B. Harvey (1940). The molarity series was M/10, M/20, M/40, M/100, M/200, M/1000, M/5000, and M/10,000 corresponding in concentration equivalents to 2.0, 1.0, 0.50, 0.20, 0.10, 0.02, 0.004, and 0.002 per cent of the alkaloid (caffeine Merck U.S.P., $C_8H_{10}O_2N_4 \cdot H_2O$, mol. wt. 212.21). This same procedure was followed by the author (1946-a) in obtaining preliminary data.

The experimental series to determine the relative sensitivity of the several stages (FM through pluteus) to a caffeine environment, was conducted in the following manner. Fertilized eggs were allowed to develop under normal conditions in SW to the desired stages. Then several hundred specimens of each of the first eight stages cited above were transferred to each of the eight concentrations of CSW. The most advanced stage of differentiation attained by the experimentals in each concentration of CSW, and the rate and total time required to reach this degree of development, were compared with control eggs from the same female developed normally in SW throughout the 72 hours. Equivalent fluid volumes in stender dishes were maintained at the temperature of running SW. Conditions of significance, such as volumes, temperature, pH, and pressure, were constant for controls and experimentals in each series. Observations were made microscopically under a water immersion lens at 440 ×.

### Table I

*Effect of Caffeine on Fertilization and Development of Arbacia ova*

Gametes shed into medium SW or CSW for 15 min. before mixing. N = normal in time and form of development to pluteus. Key: SW = normal sea water; CSW = caffeine in SW; FM = fertilization membrane.

| % | Molarity | I Control N ♀ X N ♂ Dev. SW | II Exper. N ♀ X C ♂ SW | III Exper. C ♀ X N ♂ SW | IV Exper. C ♀ X N ♂ CSW | V Exper. C ♀ X C ♂ SW | VI Exper. C ♀ X C ♂ CSW |
|---|---|---|---|---|---|---|---|
| 0.002 | M/10,000 | N | N | N | N | N | N |
| 0.004 | M/5000 | N | N | N | N | N | N |
| 0.02 | M/1000 | N | N | N | Slight retard. in early cleavage. | N | Many abnormal gastrulae. No plutei in 72 hours. |
| 0.10 | M/200 | N | N | N or slight retard. 4-cell on but pluteus normal form. | Retard. early cleavage. Arrested in late cleavage. No blastula. Cytolysis begun in 8 to 9 hours. | Normal blastula arrested in gast. Few early plutei —prism stage. | Ditto Col. IV with sharply localized echinochrome. Disintegration. |
| 0.20 | M/100 | N | N | Time delay but plutei normal form. | Time retarded. Most div. abnormal. Arrested 4 and 8's when controls in 64 or late cleavage. Echinochrome localized. Dead. | Essential form N. Gastrula pluteus transition retarded several hours. | Ditto Col. IV with abnormal form in the 2-celled stage. Cells unequal. |
| 0.50 | M/40 | N | Normal plutei time and form but pigment conc. noted and move slow. | Normal blast. Gastr. delay and arrest. Some cytolysis. No larva. | No FM or 1% FM. Egg oval. Pigment conc. begins in 2 to 3 hrs. and localized in 10 hrs. Then cytolysis. | Arrested in 16-cell to blast. Few abnormal gastrulae. No plutei before cytolysis. | Ditto Col. IV but develop pigment conc. in 5 to 7 hrs. Cytolysis. |
| 1.0 | M/20 | N | Normal blast. but few reach gastr. No plutei. | Similar to Col. II but many abnormal cleavage and some cytol. in 6–7 hrs. | No FM. Eggs shape abnormal. Pigment clump in all within 1 hour. Cytolysis. | Early cleavage time retarded. Never beyond 16. Mostly less and abnormal. | Ditto Col. IV. |
| 2.0 | M/10 | N | Time retard. 10% dev. into blast. | Time retarded. 16-cell maximum. Form abnormal. Arrested mostly in 4-celled. Pigment conc. Cytolysis. | No FM and ditto Col. IV for 1% given above. | Ditto Col. III but many arrested earlier in 2-cell. Maximum 8-celled rare. | Ditto Col. IV. No FM. |

## RESULTS

Immersion of either or both the egg and sperm in any concentration of caffeine for 15 minutes prior to mixing the gametes did not render the egg non-fertilizable nor destroy the ability of the sperm to fertilize. These gametes, however, were not unaffected, at least by the higher concentrations, since with pretreated gametes (i.e. caffeinized eggs × caffeinized sperm), although they formed the FM when mixed and allowed to develop in SW, produced a zygote which never survived be-

TABLE II

*Sensitivity to Caffeine Concentration*

| Dev. stage when transferred to caffeine | Term used is maximum development before death | | | | | |
|---|---|---|---|---|---|---|
| | 0.02% | 0.10% | 0.2% | 0.5% | 1.0% | 2.0% |
| I. Fertilization membrane | Gastrula to pluteus. Time retarded but form normal. | Blastula majority. Some gastrulae. | 8 cells. | FM. No further dev. | FM. No dev. | FM. No dev. |
| II. Streak | Gastrula to pluteus. Time retarded but form normal. | Blastula majority. Some gastrulae. | 16 cells. | No further dev. | No dev. | No dev. |
| III. 2-cells | Gastrula to pluteus. Time retarded but form normal. | 32-celled and a few blastulae. | 8 and 16. | No further dev. | No dev. | No dev. |
| IV. 4-cells | Gastrula to pluteus. Time retarded but form normal. | Blastulae fragmented badly. | 16 and a few 32. | No further dev. | No dev. | No dev. |
| V. 8-cells | Gastrula to pluteus. Time retarded but form normal. | Blastulae less fragmented and a few gast. | 16 and a few 32. | No further dev. | No dev. | No dev. |
| VI. Late cleavage | Gastrula to pluteus. Time retarded but form normal. | Blastulae. | Blast. move slow. | No further dev. | No dev. | No dev. |
| VII. Blastula | Gastrula to pluteus. Time retarded but form normal. | Gastrulae and "prism" triangles. No plutei in 3 days. | Gastr. | Abnorm. gastr. | Abnorm. gastr. Frag. badly. | No dev. Died in 3 hrs. |
| VIII. Gastrula | Gastrula to pluteus. Time retarded but form normal. | Triangular "prisms" only. No plutei, not even short prongs. | No dev. | No dev. | No dev. | No dev. |

yond the early cleavage stages. Table I reveals the fact that typical plutei developed in normal time and form in all six combinations listed under columns I–VI inclusive with regard to molarities of M/5000 and M/10,000. The final line in Table I shows that the gametes, when shed into M/10 CSW (which approaches the maximum solubility concentration of this alkaloid in SW), and allowed to develop in SW, did form the FM but the zygote was retarded in its development and failed to form plutei before death. When shed into M/10 CSW and mixed for development also in CSW, the FM did not form, or if it did, it was not separated from the egg so as to be visible. Table I presents the various conditions under which the "first" retardation effects of any specific kind were observable. This table also shows the extent to which each concentration of caffeine interfered with the symmetry of normal growth.

The ability of the organism to carry on its normal metabolism in the presence of this trimethylated purine molecule is demonstrated by Table II, the Sensitivity Table. Eight typical stages of Arbacia development were transferred to a graded series of eight different concentrations of CSW. Table II lists only six molarities since it had been found in preliminary experiments by the author (1946-b) that M/10,000 and M/5000 CSW caused no response regardless of the organism's developmental stage when placed in CSW. All stages from FM through the Gastrula continued development to normal plutei in M/1000 CSW but the time schedule was retarded. The 2- and 4-celled stages were delayed more than the subsequent cleavage stages. M/200 CSW inhibited plutei formation from all stages. The maximum effect due to this concentration was similarly on the 2-celled stage and very few individuals ultimately exceeded the 32-celled stage. Other stages reached blastulation in M/200 CSW. Gastrulae formed "prisms" but no plutei. Higher molarities, M/100, M/40, M/20, and M/10 CSW, were more retarding and the last three (M/40, M/20, M/10 CSW) typically inhibited *all* further development beyond the stage attained when subjected to caffeine.

## DISCUSSION

Data by the author (1945) on the effect of caffeine upon the oxygen consumption of the fertilized Arbacia ova, recorded evidence that caffeine does affect the normal metabolism of the zygote. The effect of the molarities less than M/200 CSW in depressing the $O_2$ uptake of fertilized Arbacia eggs was slight and variable within 10 per cent, so they were not considered significant. Repetition of these experiments has emphasized again that molarities of M/200 CSW or above clearly depress the $O_2$ uptake as shown by Figure 1, the $O_2$ consumption—time relationship graph. These same molarities are the concentrations which are unquestionably effective in affecting the fertilization and the subsequent developmental time factor in the current study. In complete agreement are the effects of increasing concentrations of caffeine in depressing the $O_2$ uptake and in retarding the developmental time. The inhibition of the $O_2$ consumption effect of caffeine is undoubtedly a primary factor in the retardation of metabolism and cell division demonstrated by Table I. The possible mechanism by which caffeine interefers with the cellular metabolism, possibly by affecting only a single site in the pathway of the cytochrome oxidase-cytochrome chain of reactions, has been discussed previously by the author (1945).

There is some evidence that the egg is more sensitive than the sperm to caffeine. It will be noted by an examination of Table I that the lowest concentration, M/1000, at which any effect can be observed is under conditions where the caffeine-pretreated egg was fertilized by a non-treated sperm and developed in a CSW environment. The first effect with M/200 CSW involves the C♀ in M/200 CSW × N♂ and development in SW. The reverse, N♀ × C♂ M/200 CSW, with development in SW caused no retardation effect.

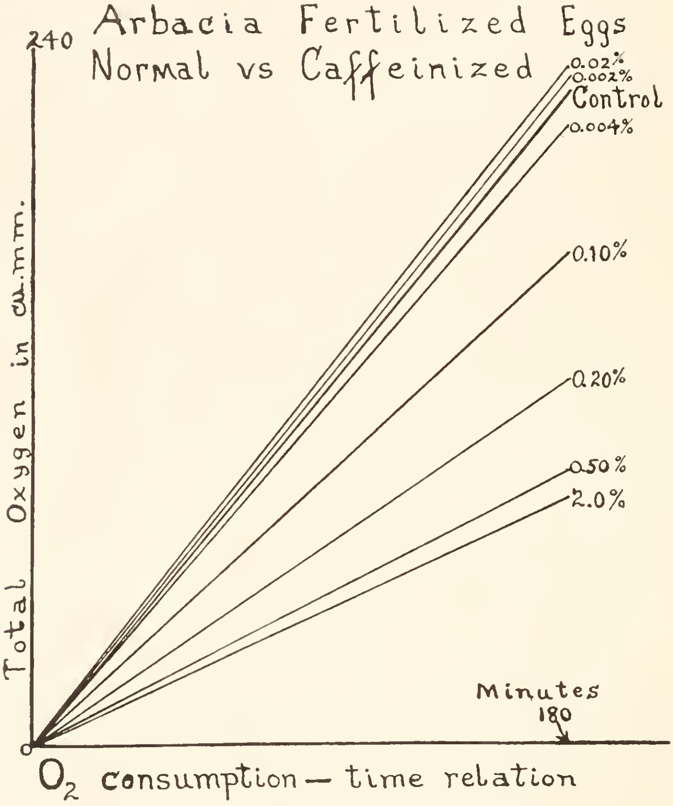

FIGURE 1

An osmotic effect of caffeine might be a cause underlying the sensitivity effects observed. Consequently, the eggs of a single female were subjected to the concentration series of the eight different molarities. Temperature was uniform by keeping the stender dishes in running sea water. The pH was not a factor since the differences between the normal SW, van't Hoff's artificial SW, and caffeine in either, did not increase the alkalinity more than 0.06 of a pH unit even in the highest concentration employed. This is well within the range, 6.0 – 8.3, within which the Arbacia egg is fertilizable and normal development occurs as shown by Smith and Clowes (1924). Since the unfertilized Arbacia egg is a sphere, egg diameters were measured directly by an ocular micrometer and volumes were calculated by the formula, $r^3 \times 4/3\pi$. In the case of the unfertilized egg alone, a total of over 5000 ova in the eight experimental media were examined. Detailed results employing the most drastic concentration (2 per cent) are cited in Table III.

Obviously, no significant effect can be concluded from the facts presented in Table III for the unfertilized egg. Similar statistics indicated also negative results with reference to the fertilized egg. A slight osmotic effect with time, if due to caffeine, could conceivably alter the transport of water which could modify the internal metabolism of the egg.

TABLE III

*Diameter and volume variation in caffeinized* (2% ≈ *M/10*) *Arbacia eggs*

| Egg No.* | 30 *Minutes* after shedding eggs into | | | | 2 *Hours* after shedding eggs into | | | | 7 *Hours* after shedding eggs into | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sea water | | 2% Caffeine-in-SW | | Sea water | | 2% Caffeine-in-SW | | Sea water | | 2% Caffeine-in-SW | |
| | Diameter in micra | Volume in cu. micra ×1000 | Diameter in micra | Volume in cu. micra ×1000 | Diameter in micra | Volume in cu. micra ×1000 | Diameter in micra | Volume in cu. micra ×1000 | Diameter in micra | Volume in cu. micra ×1000 | Diameter in micra | Volume in cu. micra ×1000 |
| 1 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 72.6 | 201 | 71.0 | 187 | 72.6 | 201 |
| 2 | 72.6 | 201 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 72.6 | 201 | 72.6 | 201 |
| 3 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 72.6 | 201 | 72.6 | 201 |
| 4 | 72.6 | 201 | 71.0 | 187 | 72.6 | 201 | 72.6 | 201 | 71.0 | 187 | 72.6 | 201 |
| 5 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 72.6 | 201 | 71.0 | 187 | 74.3 | 214 |
| 6 | 69.3 | 173 | 71.0 | 187 | 72.6 | 201 | 71.0 | 187 | 72.6 | 201 | 74.3 | 214 |
| 7 | 71.0 | 187 | 72.6 | 201 | 72.6 | 201 | 71.0 | 187 | 72.6 | 201 | 71.0 | 187 |
| 8 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 72.6 | 201 |
| 9 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 72.6 | 201 | 72.6 | 201 | 71.0 | 187 |
| 10 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 | 71.0 | 187 |
| Aver. | 71.2 | 188 | 71.2 | 188 | 71.5 | 191 | 71.6 | 193 | 71.8 | 194 | 72.5 | 199 |
| | 30 Min. normal vs. caffeinized (2%)<br>Diameter difference is zero<br>Volume difference is zero | | | | 2 Hr. normal vs. caffeinized (2%)<br>Diameter difference is 0.1 mu<br>Volume difference is 1400 = 0.73% increase | | | | 7 Hr. normal vs. caffeinized (2%)<br>Diameter difference is 0.7 mu<br>Volume difference is 5300 = 2.75% increase | | | |

No significant variation in 30 minutes, two hours, and seven hours.

* Each egg diameter listed is the average of 10 micrometer unit measurements on eggs from a single female. Final average of the 10 diameters cited in each column is actually the average of 100 eggs from the same female.

Advantage was taken of the procedures contributed to the literature by Harvey (1910), Lillie (1916, 1917, 1918), McCutcheon and Lucké (1926), Stewart (1931), and Jacobs and Stewart (1932), all of whom studied permeabilities employing the Arbacia egg as test material. Since rapid dehydration by strongly hypertonic solutions has destructive action on fertilized eggs, hypotonic solutions were used from which temporary immersion (30 minutes) results in complete recovery if transferred to normal SW.

Eggs from the same female were employed for both the unfertilized and fertilized series of experimental conditions. Several hundred ova, according to the series, were placed in stender dishes of normal SW (referred to here as NSW), van't Hoff's artificial sea water (ASW), bicarbonate—buffered to the pH of the NSW, hypotonic (40 per cent) sea water (40 ASW: 60 distilled water) indicated here as HSW, and HSW plus caffeine, i.e. CSW. The caffeine concentrations used were M/1000 (0.02 per cent), M/100 (0.20 per cent), and M/10 (2.0 percent). At the end of the first 30-minute period in any given experiment, approximately half of the eggs were transferred from CSW and divided approximately into equal masses to NSW and ASW for recovery studies. Readings (diameters) were made each minute for 10 minutes, followed by readings at 20, 30, and 60 minutes, and periodically for 24 hours although only the first 60 minutes were significant in the current study. Statistical tables similar to Table III were compiled. Molarities of M/1000, M/100, M/10 CSW in 40 per cent ASW were chosen because they represent significant concentrations as indicated by retardation effects of caffeine in Arbacia development—see Table I.

Since osmotic swelling is primarily one of diffusion, it is expected that when a cell is far from osmotic equilibrium with its environment, it will swell rapidly but the rate of swelling will decrease as equilibrium is approached. Volume changes were plotted against time. Thereby the velocity of swelling was noted. The rate of swelling was determined by the formula (McCutcheon and Lucké, 1926) in general use for this purpose; namely,

$$Kt = l_n \frac{V_{eq} - V_0}{V_{eq} - V_t}.$$

The experimental points were found to fall along a straight line as would be expected when

$$\log \frac{V_{eq} - V_0}{V_{eq} - V_t}$$

was plotted against time. The slope of the line gives the value of the velocity constant as $k$. This equation expresses the process of swelling in 40 per cent HSW and 40 per cent CSW for both unfertilized and fertilized eggs. This would be anticipated in a diffusion process.

The comparison of primary interest in the current paper is between data derived from the osmotic changes (rate and amount) specifically in HSW and the corresponding CSW. Comparison of these effects was also compared with the controls in NSW and ASW. No significant difference was evident in unfertilized eggs under the two hypotonic media of HSW and the corresponding CSW for any of the three concentrations of caffeine. Fertilized eggs show no difference under these experimental conditions. True, the fertilized eggs were more permeable than unfertilized eggs but the addition of caffeine had relatively no effect upon either series. *In other words, the HSW and the corresponding CSW results were equivalent.* Greater permeability to water and greater variability of fertilized eggs was expected since there is relative constancy of $O_2$ consumption before fertilization. The concentrations used have been shown earlier in this paper by the author to retard or inhibit cleavage completely. This fact indicates that the changes occurring in permeability at the time of cleavage are of a different kind from those associated with cleavage furrow formation per se. The latter process is affected by caffeine which retards the $O_2$ uptake (Cheney, 1945).

The possibility of a viscosity effect of caffeine upon the Arbacia egg protoplasm, as a factor in explanation of the observed retardation of cleavage in caffeine solutions, has led to a study of the echinochrome stratification and egg deformation by centrifugation. The report upon these phenomena and the effect of caffeine upon the tension forces at the surface layers will be discussed in a later publication.

## SUMMARY

1. Caffeine does affect the gametes and the zygotes development of *Arbacia punctulata*.

2. Pre-treatment of both gametes with caffeine (0.5 per cent or higher) prior to mixing for fertilization and developed in CSW prevents the separation of the fertilization membrane.

3. Since some development occurs if both gametes are caffeinized but developed in SW, the FM may form but not be separated from the egg mass in the case of CSW as the environment.

4. The results of gamete activity following immersion and subsequent fertilization and development in CSW suggest that the egg is somewhat more sensitive than the sperm to caffeine.

5. Minimum effective concentrations in general are 0.1 per cent CSW (M/200). Slight retardation of development in 0.02 per cent CSW, under conditions of C♀ × N♂ and development in SW, is noted.

6. Oxygen uptake inhibition effect of caffeine is a primary factor in the retardation of cleavage time.

7. Early cleavage stages are retarded most.

8. The inhibition of $O_2$ utilization, retardation of cleavage time, and the degree of interference with the symmetrical differentiation attained, are directly proportional to the caffeine concentrations.

9. Concentrations of 0.5 per cent CSW (M/40) or higher as an environmental medium inhibit completely the physiological processes underlying the mechanics of further differentiation.

10. Caffeine does not initiate an osmotic effect with regard to the transport of water into the Arbacia egg.

11. Other factors in the explanation of the results observed are suggested.

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# THE RESISTANCE OF METALLIC SILVER TO MARINE FOULING

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Copper has been commonly employed to resist the attachment of fouling growths on structures exposed to the sea, while the salts of both copper and mercury are commonly employed as toxic pigments in antifouling paints. The toxicity of solutions of silver salts to organisms such as barnacles has been shown by Clarke (1947) to be comparable to that of copper and mercury. Mr. F. L. LaQue of the International Nickel Company informs us that specimens of fine silver which he exposed at Kure Beach, North Carolina, for as long as 195 days remained practically free of fouling. Similar results were observed by Dr. W. F. Clapp with exposures at Marion, Massachusetts. On the other hand, the Kure Beach specimens when cleaned and re-exposed at Duxbury, Massachusetts, fouled as heavily as nickel or other inert metals tested at the same time. The reason for this difference in behavior is obscure. Mr. LaQue (in press) has classified silver among metals with variable or intermediate ability to resist fouling.

Because of the uncertainty existing concerning the fouling of silver, Mr. LaQue suggested that more systematic tests should be conducted and with the help of Mr. J. L. Christie of the Handy and Harman Company supplied the specimens used in the study to be reported. These consisted of fine silver containing no copper and of coin silver in which 10 per cent of copper is present as an alloy.

In the first experiment, summarized in Table I, two panels of pure silver, one panel of coin silver and one of copper were exposed simultaneously in the sea at Miami Beach, Florida, for one year. One of the silver panels had previously been exposed for two months to running sea water in the laboratory under conditions which permitted a black coating of corrosion products (presumably silver chloride) to form on the surface. The sterling silver and copper panels were 4 × 6 inches in dimensions, the coin silver 3 × 3 inches. The panels were held in a wooden frame which prevented metallic contact between them, and left almost the entire area of both sides exposed to the sea water.

Throughout the year's exposure the coin silver remained entirely free of fouling. It resisted attachment better than the sample of copper to which light fouling, consisting of barnacles and encrusting bryozoans, attached temporarily from time to time during the exposure. The copper, however, was entirely clean at the eleventh month, after which the specimen was lost. In contrast, the two specimens of pure silver commenced to foul within the first and second month and both were completely covered at the end of the fourth month of exposure.

One specimen of silver had been soaked in sea water prior to exposure in the sea in the expectation that fouling would be accelerated by the formation of a coating of corrosion products on its surface. This expectation was not realized. The

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[1] Contribution No. 411.

Table I

*Monthly fouling ratings on silver, copper and a silver alloy on marine exposure at Miami Beach, Florida, March 1, 1946–March 1, 1947*

| Months. . . . . . . | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Silver | 3 | 2 | 2<br>1 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Silver presoaked 2 months | 0 | 1 | 2<br>0 | 4 | 4 | 4 | 4<br>3 | 4 | 4 | 4 | 4 | — |
| Coin silver | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Copper | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | — |

*Fouling Ratings*

0 = no fouling
1 = <25% fouled
2 = 25–50% fouled
3 = 50–75% fouled
4 = 75–100% fouled

A double rating indicates a different degree of fouling for the two sides of a panel.

panel actually resisted fouling during the early months of exposure better than the specimen immersed with a clean surface of bright metal.

The resistance of copper to fouling was attributed by Sir Humphrey Davy (1824) to the solution of the metal. Subsequent studies (Ketchum, Ferry, Redfield and Burns, 1943) led to the belief that the ions formed on the solution of copper or its salts prevent the attachment of marine organisms by virtue of their poisonous action . Davy's conclusion was based primarily on the observation that copper did not foul when, through contact with a baser metal, a galvanic couple was formed which prevented the solution of the copper. In discussing the germicidal effects of silver, Goetz, Tracy and Goetz (1942) have pointed out that one must distinguish between the volume effect due to small silver ion concentrations in the water and the surface effect which occurs when microorganisms are brought into wetting contact with surfaces on which silver is absorbed. In anticipation of the possibility that metallic silver might resist the attachment of fouling organisms by virtue of some surface effect which would persist after solution was suppressed by galvanic action, a series of silver panels were exposed to the sea coupled with several metals standing higher in the galvanic series.

In this experiment, which is summarized in Table II, strips of copper, nickel, iron, and zinc, 1 × 6 inches in dimensions, were held in contact with 4 × 6 inch silver panels by means of the supporting rack. One uncoupled panel of pure silver was included as a control, as was a silver dollar, as a further check on the fouling of coin silver.

The results confirmed the other experiment in that the silver coin resisted fouling for the entire period of twelve months' exposure except for the temporary attachment of some early larval stages (incipient fouling) during the first two months.

### TABLE II

*Monthly fouling ratings of silver coupled with various metals on marine exposure at Miami Beach, Florida, January 1, 1946–January 1, 1947*

| Months.... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ag | 1 | 1 | 1 | 1 | 4* | 4* | 4* | 4* | 4* | 4* | 4* | 4* |
| Ag–Cu | 1 | 1 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Ag–Ni | 2 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Ag–Fe | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Ag–Zn | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Ag–dollar | ½ | ½ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* Principally a sponge covering entire panel and excluding other fouling from attaching.

*Fouling Ratings*

0 = no fouling
½ = incipient fouling
1 = <25% fouled
2 = 25–50% fouled
3 = 50–75% fouled
4 = 75–100% fouled

The pure silver, in contrast, was fouled lightly during the first four months after which it became generally covered with an encrusting sponge.[2]

The effect of coupling the silver to a baser metal was to shorten the time required for the silver to foul. When coupled with the more electro-negative metals, zinc and iron, the silver fouled heavily during the first month. Coupled to nickel the silver showed some slight resistance to fouling only during the first two months. Contact with copper had scarcely any effect on the fouling of the silver. The effects are thus proportional to the difference in potential of the coupled metals.

These results provide a useful control on the resistance of silver to fouling since they show that the light fouling of the uncoupled silver during the early months of exposure are not due to conditions unfavorable to fouling during this period. They warrant the conclusion that the temporary resistance of silver to fouling is dependent on the solution of the metal in the same sense as is the case with copper. There is no indication that the surface of silver, from which solution is prevented by coupling with zinc or iron, has any antifouling action. It should be pointed out, however, that these observations do not preclude in any way the possibility that surface effects such as discussed by Goetz, Tracy and Goetz (1942) might be influential in preventing attachment of fouling if suitable surfaces were established.

The most interesting and perhaps useful result of these experiments is the demonstration of the prolonged resistance to fouling of coin silver, which is apparently equal to copper and superior to pure silver. Coin silver contains about 10 per cent of copper, an amount much less than is present in other copper alloys which successfully resist fouling. The explanation appears to be in the high rate of solution of the coin silver when exposed in the sea.

The specimen of coin silver exposed at Miami lost weight during the year at an average rate of 16.8 mg/dm$^2$/day, whereas the specimen of pure silver simultaneously exposed corroded at an average rate of 5.6 mg/dm$^2$/day. Thus, the rate of solution of the silver was three times as great in the case of the coin silver, which in itself may account for its more prolonged resistance to fouling. Moreover, since copper composed 10 per cent of the alloy, copper may be assumed to have been liberated at a rate of 1.68 mg/dm$^2$/day. While this is a lower rate than is adequate to prevent fouling in the case of other copper alloys, it exceeds the rate of solution (1 mg/dm$^2$/day) of copper from effective antifouling paints. It seems probable consequently that the resistance of coin silver to fouling results from the combined effects of the silver and copper ions which are liberated as it dissolves.

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[2] Mr. LaQue exposed duplicates of these specimens at Kure Beach, N. C., for 169 days during the summer of 1946. Neither the fine or coin silver showed any fouling. However, fouling at the test location was extremely light.

A duplicate specimen of coin silver, exposed to the sea at Woods Hole during the same period, lost weight at an average rate of 3.52 mg/dm$^2$/day. This is less than a quarter of the rate of solution observed in the warmer waters at Miami. It seems quite possible that under conditions of exposure which result in such low rates of solution coin silver may foul more readily. Unfortunately, little fouling occurs under any circumstances at the point of exposure at Woods Hole so that this possibility is not tested. Variation in the resistance of specimens of silver to fouling may be expected to result from variations in the conditions of exposure, such as temperature, which influence the rate of corrosion, as well as to variations in the copper present in the alloy.

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# THE LIPIDS IN COLPIDIUM CAMPYLUM

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## INTRODUCTION

Various investigators have reported that *Colpidium* contains fat in the cytoplasm. Mast (1938) holds that it is neutral fat; Wilber (1942) maintains that it is chiefly fatty acid. Both investigators, however, base their conclusions on the staining of colpidia with Nile blue sulfate.

In the literature there is apparently no information based on microchemical analyses concerning the formation and types of lipids in *Colpidium*. An investigation was consequently undertaken to ascertain the types of lipids present and the manner of formation. This present report is the first in a series and it deals with the synthesis of lipids in *Colpidium campylum*.

## MATERIAL AND METHODS

A sterile culture of colpidia was established (Glaser and Coria, 1930) and was cultivated in 3 per cent Difco proteose-peptone solution (Elliot, 1933). The proteose-peptone was rendered fat-free by extracting it with hot alcohol (Bloor, 1943, p. 40). Solutions of this fat-free medium were negative to all the tests for lipids. Colpidia were washed 3 times in fat-free proteose-peptone solution before transfer from ordinary to fat-free culture medium.

The population of the cultures was estimated by counting the number of colpidia in a small, weighed drop of medium and from that result calculating the number of organisms in 1 cc.

Five days after transfer (when the population reached about 44,000 colpidia per cc.) 1 cc. of the culture (fluid plus organisms) was transferred to 17 cc. of boiling alcohol-ether mixture and the whole was then made up to 25 cc. The alcohol-ether mixture extracted all the lipids (Bloor, 1943). Phospholipids were estimated colorimetrically (Youngburg and Youngburg, 1930), cholesterol colorimetrically (Bloor, 1916), and fatty acid by oxidation (Bloor, 1928). The procedure is somewhat as follows: For estimation of neutral fat and bound cholesterol the extracted material is saponified; this sets free the cholesterol and the fatty acids contained in fat and cholesterol esters. Tests for free cholesterol were made and were consistently negative. Since no cholesterol was found, the fatty acid values represent fatty acid combined in fat and/or free in the extract. It should be remembered that "the cholesterol esters are normally present only in traces. Hence, after phospholipids are separated, the fatty acids present may be safely referred to as fats." (See Bloor, 1943, pp. 42–43.)

A second sample from the same culture was carefully filtered 3 times through fat-free cotton waste and hard filter paper to remove all the colpidia. The filtrate was then analyzed for lipid material.

The differences between the values for the whole culture and for the filtrate were equal to the amounts of lipids in the colpidia themselves.

It is extremely rare that pure fats are found in microorganisms. Usually they are found as mixtures which are almost impossible to separate. "Analyses, therefore, are restricted usually to the fatty acids present and the determination of the ratio of saturated to unsaturated fatty acids" (Porter, 1946, p. 409). This method was followed in the present work, although the degree of saturation of the fatty acids was not measured.

## RESULTS

All tests for cholesterol were negative. Table I shows that there is consistently about .0002 mg. of lipid phosphorus per 1,000 colpidia. The table also shows that there is more lipid phosphorus in the culture fluid (2.5 mg./100 cc.) than in the colpidia (0.9 mg. in the colpidia from 100 cc.).

TABLE I

*Table showing the amount of lipid phosphorus in the culture medium and in the colpidia*

In order to convert lipid *P* to phospholipid (assuming it all to be lecithin) multiply the lipid *P* values by 25.

| Culture | Colpidia per cc. | Lipid *P* in whole culture. Mg./100 cc. | Lipid *P* in fluid alone. Mg./100 cc. | Lipid *P* in colpidia from 100 cc. Mg. | Lipid *P* in 1,000 colpidia. Mg. |
|---|---|---|---|---|---|
| 101 | 44,000 | 3.6 | 2.3 | 1.3 | 0.0003 |
| 102 | 47,000 | 3.3 | 2.3 | 1.0 | 0.0003 |
| 103 | 39,000 | 3.3 | 2.6 | 0.7 | 0.0002 |
| 104 | 47,000 | 3.6 | 2.8 | 0.8 | 0.0002 |
| 105 | 44,000 | 3.1 | 2.3 | 0.8 | 0.0002 |
| Mean | 44,000 | 3.4 | 2.5 | 0.9 | 0.0002 |

TABLE II

*Table showing the amount of fatty acid in the culture medium and in the colpidia themselves*

| Culture | Colpidia per cc. | Lipid in culture. Mg./100 cc. | Lipid in fluid alone. Mg./100 cc. | Lipid in colpidia from 100 cc. Mg. | Lipid in 1,000 colpidia. Mg. |
|---|---|---|---|---|---|
| 101 | 44,000 | 212 | 150 | 62 | 0.012 |
| 102 | 47,000 | 225 | 187 | 38 | 0.008 |
| 103 | 39,000 | 225 | 175 | 50 | 0.013 |
| 104 | 47,000 | 263 | 213 | 50 | 0.010 |
| 105 | 44,000 | 237 | 188 | 49 | 0.011 |
| Mean | 44,000 | 232 | 183 | 49 | 0.011 |

Table II shows that there is consistently about 0.011 mg. of fatty acid per 1,000 colpidia. This table also shows that there is much more fatty acid in the culture fluid (183 mg./100 cc.) than in the colpidia (49 mg. in the colpidia from 100 cc.).

## DISCUSSION

At first sight the value of 0.011 mg. of fatty acid in 1,000 colpidia seems large. Calculations were made to ascertain roughly the percentage of the wet weight which is lipid. It was assumed that *Colpidium* is a prolate spheroid. The volume of the individual organism is then expressed: $V = 4/3\pi a^2 b$, in which $a$ is the minor semi-axis and $b$ the major semi-axis.

The mean length of 20 colpidia selected at random from the cultures was 0.064 mm., the mean thickness 0.036 mm. The minor and major semi-axes are then 0.018 mm. and 0.032 mm., respectively. Substituting these values in the equation above we get $V = 0.0000418$ mm$^3$ for a single colpidium. For 1,000 colpidia, $V =$ 0.0418 mm$^3$.

Assuming the specific gravity of protoplasm to be 1.00, the weight of 1,000 18 colpidia is 0.0148 mg. The amount of lipid is then 24 per cent of the wet weight.

For the first time quantitative estimates of the kind and the amount of lipid in *Colpidium* are presented. The apparent absence of cholesterol is in accord with other investigations on the protozoa (Wilber, 1946 a and b). The evidence strongly suggests that colpidia do not synthesize cholesterol. Whether they assimilate cholesterol, if it is present in the culture medium, is not known. The high content of fatty acid is in accord with results obtained with other micro-organisms: the lipid in the diphtheria bacillus is mostly fatty acid (Chargaff, 1933); all the lipid in the typhoid bacillus is fatty acid (Akasi, 1939); in myobacterium fatty acids make up about 70 per cent of the fat (Pangborn, Chargaff, and Anderson, 1932).

The production of fat (sudanophil granules) from ingested starch and protein has been reported in various ciliates (Zingher, 1933). However, neither fat free nor sterile culture media were used in the investigations.

It is interesting to observe that the colpidia produce such large amounts of lipid that some is eliminated into the surrounding culture fluid. In the present experiments the ratio of *phospholipid in the whole culture/phospholipid in culture fluid alone* is 1.2 and that of *fatty acid in the whole culture/fatty acid in the culture fluid alone* is 1.3. The inverse ratios are 0.7 and 0.8 respectively. All the cultures used were approximately the same age and contained about the same density of population. It is, therefore, not possible at present to conclude whether these ratios are constant or whether they vary with age of culture and size of population. Such an investigation is now in progress.

In the higher organisms, it is known that fat is formed from protein (Atkinson, Rapport, and Lush, 1922; Eckstein, 1929). The present results indicate that there is a synthesis of lipid from protein in *Colpidium* also. The medium in which the organisms were grown contained no measurable lipids. The cultures were completely sterile, as indicated by regular bacteriological tests. After colpidia had grown in the fat-free medium there was appreciable lipid material in the organisms and in the culture fluid. The only source of food for the colpidia was protein (the proteose-peptone). It seems clear, therefore, that the growing colpidia broke down the supplied protein and reformed it into the lipids which were demonstrated by chemical analysis.

## SUMMARY

1. *Colpidium campylum* was established in sterile culture and grown in fat-free proteose-peptone solution.

2. The colpidia and the culture fluid in which they were grown were analyzed for fatty acid, cholesterol, and phospholipid.

3. No cholesterol was found. Large amounts of fatty acid were found in the organisms and in the ambient culture fluid. Phospholipid was found in the colpidia and in the culture fluid. There was more lipid in the culture fluid than in the organisms.

4. It is concluded that *Colpidium* synthesizes lipid from protein.

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# SURFACE ILLUMINATION AND BARNACLE ATTACHMENT

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During the past twenty years the effect of light conditions upon the attachment of barnacles has received a considerable amount of attention. The investigations of Visscher (1928) have shown that barnacles attach more readily to dark-colored than to green, white, or other light-colored surfaces. This has since been confirmed by Edmondson and Ingram (1939). Pomerat and Reiner (1942) report greater numbers of attachments of *Balanus eburneus* to the underside of horizontal black plates than to opal plates during daylight. Fewer numbers attached to the upper surface of black plates than to the lower surface. The distinction between black and opal was not apparent during darkness. These authors also found that attachment to opal and transparent collectors were similar on both upper and lower surfaces. Confirmatory results were obtained by Pomerat and Gregg (1942) and by Edmondson (1944).

The observations of McDougall (1943) substantiate those previously mentioned. Nevertheless, although during the day time the rate of attachment was greater in shade than in sunlight, it was considerably lower at night upon any surface. This might suggest that the stimulus to attachment was not a uniform condition of relative shade but rather the contrast in lighting conditions present only during daylight. Nevertheless, attempts by Gregg (1945) to demonstrate a stimulus to attachment in the contrasting effect of adjacent light and dark surfaces were inconclusive.

Since 1942 investigators at Miami have repeatedly observed, in the case of *Balanus amphitrite niveus* Darwin and *B. improvisus* Darwin, not only a greater rate of attachment to darker surfaces during daylight, but also a diminished rate of attachment to all surfaces during the hours of darkness. Furthermore, attachments in darkness were found to be even less than attachments to light colored surfaces during daytime.

In the absence of the last mentioned observation, the lesser attachment to lighter colors in daylight and the equal attachments to all surfaces during darkness might be explained in terms of a negative phototropotaxis, using the terminology of Fraenkel and Gunn (1940). This would bring about movement away from light surfaces in daylight and would account for failure to distinguish any particular surface during night time. It would not, however, explain the greater number of attachments to *light* surfaces in daylight than in darkness. The theory of negative phototropotactic behavior is apparently supported by the laboratory observations of Runnstrom (1925), Visscher (1928b), Visscher and Luce (1928), Hertz (1933) and Neu (1933), who show that while the nauplius larvae swim towards the lighted side of a vessel, cyprid larvae swim away and attach on the opposite side, their activity being greater in green light than in other portions of the spectrum.

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[1] Contribution No. 20 from the Marine Laboratory, University of Miami.

Observations communicated by Gregg (in literis) show that under conditions of weak artificial illumination at night, attachments to white plates are greater than to black, thus reversing the daytime condition. This and the previously mentioned anomalies, which are not readily explained by simple negative phototropotaxis, prompted the experiments here described, whereby the numerical distribution of cyprids between adjacent black and white surfaces of various sizes in daylight was investigated as a preliminary to further observations under carefully controlled artificial illumination.

Acknowledgments are due to Mr. James H. Gregg for his valuable assistance in setting up the experiments.

## METHODS

The apparatus consisted of a series of similar glass plates eight inches square, painted checkerboard fashion with alternating black and white squares of equal sizes. The size of the squares varied from the extreme case in which the plate was painted entirely black or entirely white, to the other extreme, where the plate was painted with 64 one-inch squares. The plates were suspended in a wooden frame just below the surface of the sea water at the Miami Beach Boat Slips in such a way as to lie in a vertical plane parallel to the direction of tidal currents.

The experiments were repeated at two different sites, one inside a covered dock and the other in the open at the side of a dock. In the latter case, one side of the rack holding the panels was facing the open sea and the other was facing the darker underside of the dock. The arrangement of plates with respect to each other was a random one determined by removing numbered cards from a hat. By painting the reverse side of each plate with a different system of squares a different sequence of plates was provided on the two sides of the rack, thus providing two sets of observations.

The rack of plates was suspended in the covered dock from 9 A.M. to 5 P.M. on a calm, sunny day. On the following day, under the same weather conditions, it was exposed for a similar period in the open dock so that a total of four sets of observations was taken. Counts of the attached cyprids were made immediately upon removing from the water.

## RESULTS

Individual observations are recorded in Table I and the average of the four exposures is recorded at the end of the table. In the average of four exposures, the average density of attachments upon the entire surface of each panel, including both black and white areas, varied from the panel of one-inch squares with 5.7 per square inch to the panel of four-inch squares with 4.7. The attachments on an equal area of the panel consisting of an eight-inch black square were 4.6, Figure 1. The checkered panels as a whole, therefore, acquired attachments somewhat greater in density than the single black panel, with a decrease in average density as the size of squares increased. On the plain white panel the average attachment density was 1.4 per square inch, or considerably less than in the case of the checkered or black panels.

Table I shows that with decreasing numbers and increasing size of squares the numbers of attachments to white surfaces decreased. Thus, on the plate with one

TABLE I

*Variation in density of barnacle attachments upon black and white squares of checkerboard collectors in relation to size of squares*

Expressed in attachments per square inch

| Experiment | Surface | Size of squares | | | |
|---|---|---|---|---|---|
| | | 8 inch entirely black or white | 4 inch | 2 inch | 1 inch |
| Covered dock<br>Side A<br>2/16/44 | Black | 4.4 | 5.8 | 4.1 | 4.6 |
| | White | 0.7 | 2.2 | 4.4 | 4.6 |
| | Average entire panel | 2.5 | 4.0 | 4.2 | 4.6 |
| | Ratio black/white | 6.6 | 2.6 | 0.9 | 1.0 |
| Covered dock<br>Side B<br>2/16/44 | Black | 3.6 | 6.1 | 6.0 | 5.5 |
| | White | 1.2 | 2.5 | 4.8 | 4.4 |
| | Entire panel | 2.4 | 4.3 | 5.4 | 4.9 |
| | Ratio black/white | 3.3 | 2.5 | 1.3 | 1.2 |
| Open dock<br>Side A<br>2/17/44 | Black | 2.9 | 5.2 | 3.5 | 4.6 |
| | White | 1.1 | 2.2 | 4.4 | 6.0 |
| | Average entire panel | 2.0 | 3.7 | 3.9 | 5.3 |
| | Ratio black/white | 2.6 | 2.5 | 0.9 | 0.8 |
| Open dock<br>Side B<br>2/17/44 | Black | 7.3 | 8.8 | 6.4 | 6.8 |
| | White | 2.6 | 5.0 | 6.0 | 9.3 |
| | Average entire panel | 4.9 | 6.9 | 6.2 | 8.0 |
| | Ratio black/white | 2.6 | 1.7 | 1.6 | 0.8 |
| Average of 4<br>exposures | Black | 4.6 | 6.4 | 5.0 | 5.4 |
| | White | 1.4 | 3.0 | 4.9 | 6.1 |
| | Average entire panel | 3.0 | 4.7 | 4.9 | 5.7 |
| | Ratio black/white | 3.3 | 2.2 | 1.0 | 0.9 |

inch squares, the average number of attachments to white areas was 6.1 per square inch. The density of attachment to an equivalent area on the plain white plate, consisting of one eight-inch square only, was 1.4. This is less than one quarter of the attachment to one-inch squares. On the plates with two- and four-inch squares the attachments were intermediate in number.

Attachments to black squares showed less variation. The attachment was greatest on the 4-square panel with four-inch squares, where the average density was 6.5, and least on the plain black eight-inch panel, with an average of 4.6 per square inch, or about three-quarters of the attachment to four-inch squares. Thus not only was variation considerably less than in the case of white squares, but it did not bear a continuous relation to the size of squares, and may not be significant.

It follows from the variation in attachments to white squares that the ratio of attachments upon black areas to attachments upon white areas decreased with diminishing size of squares. It was greater than 3 on the eight-inch squares whereas on the one- and two-inch squares black and white attachments were almost equal.

In order to demonstrate the absence of simple tropotactic response to surface illumination, the ratio of attachments to black over attachments to white is plotted

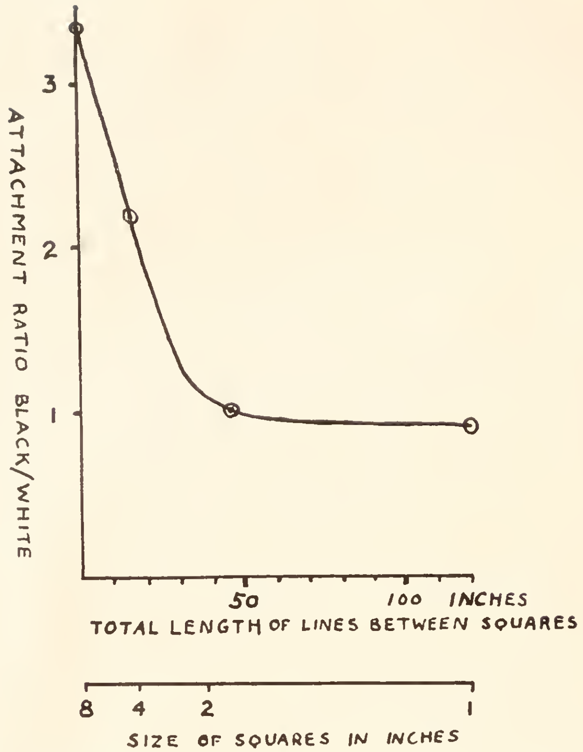

FIGURE 1. Ratio of attachments to black and to white squares in relation to size of squares and to length of black-white boundary lines.

in Figure 1 against the linear extent of the lines separating black and white squares on each plate. From this figure it is apparent that the ratio diminishes as the black/white separation length increases, reaching unity at a point corresponding to a square of side about 2 inches.

An examination of the data for the individual experiments shows a similar behavior in each case. The panels exposed in the covered dock did not differ significantly in numerical attachments, but showed a slightly lower attachment to large white squares, and consequently a slightly higher black/white ratio for the larger squares. It is doubtful whether the difference is sufficient to be of significance.

## DISCUSSION

It is apparent that the results do not uphold the simple conception that a cyprid swims actively towards dark surfaces only or swims away from white surfaces, or is capable of orientation so as to move towards a black surface in preference to a white surface. The results indicate rather that with increasing opportunity of exerting such a "choice" the cyprids show an increasing lack of ability to discriminate between black and white surfaces (Fig. 1). The apparent discrimination is limited to squares over 2 inches in size.

The attachments to black surfaces appear to be little affected by adjacent white surfaces except possibly in the case of four-inch squares. A greater density of attachments to black is associated with the presence of four-inch white squares rather than with black alone, or with black coupled to white squares smaller than four inches. The difference is rather small, however, and may not be significant.

The most striking result of the experiments is the very considerable decrease in attachment density on white squares as the size of squares increases. This is in harmony with the possibility that the cyprid is influenced not by directional light rays from the panel surface but by the general light flux or intensity of diffuse light at some distance from it. In this manner diffused light resulting from scattered reflections adjacent to larger white squares would be more intense and more effective than diffused light produced by reflection adjacent to the smaller white squares where the black surfaces would be sufficiently close to exert a "diluting" effect on the light flux. On the basis of this explanation the cyprids may be stimulated to attach when they enter into an area characterized by low intensity of diffuse light, or they may be inhibited from attaching to an increasing extent as the intensity of diffuse light increases. They would thus be subjected to the inhibiting effect as they approached large white squares, but smaller white squares as indicated above would be less effective because of the increasingly fine admixture of black surfaces which would provide a light flux in the immediate neighborhood approximating to that which a hypothetical gray surface would produce.

Greater attachment density to the small size checkerboards than to plain black surfaces may possibly indicate that there is an optimum intensity of diffuse light represented by that of the small square checkerboards equivalent to that produced by gray surfaces and that unrelieved black panels are less effective in providing attachments because the light intensity is below the optimum. The existence of an optimum light intensity would also explain the low attachment density characteristic of panels exposed during darkness which would be below the optimum. The anomalies reported by Gregg whereby, under conditions of weak artificial illumination at night, barnacles attach in greater numbers to white than to black panels may also be explained by the existence of an optimum intensity of light for attachment. White squares under weak artificial illumination might provide diffuse light of intensity nearer the optimum than black, whereas in daylight white would produce light above the optimum, black slightly below the optimum, and small checkerboards or gray would produce approximately the optimum. It is unfortunate that conditions did not permit an accurate determination of the illumination. It may be possible to remedy this in future experiments in which it is proposed to provide controlled artificial illumination during hours of darkness.

The experiments of Visscher and others (op. cit.) have demonstrated a simple negative phototropotaxis under laboratory conditions which is not borne out by the present investigations. The apparent contradiction, however, may be readily resolved when the natural conditions of submarine illumination are taken into consideration. As Whitney (1941) and Schallek (1942) have pointed out, light is usually polydirectional under sea water and only rarely is there a truly unidirectional beam. The sediment present under coastal and estuarine conditions where barnacles naturally occur serves to intensify the diffuse nature of the light. While working in diving helmets in 15 feet of water at the site of the present experiments it was found most difficult to orient with respect to the shaded and open part of the dock. The intensity of illumination appeared to be almost equal in every direction, yet after taking a few steps into the shade of the dock the intensity would suddenly decrease very considerably. Even at a point immediately below the edge of the dock little difference was experienced in the light intensity when facing towards or away from the covered area.

It is possible that other and more reasonable interpretations of the experiments presented here may exist, but it is believed that the results, at least, indicate the necessity of a renewed examination of the light reactions of barnacle cyprids during attachment, preferably by means of carefully controlled laboratory experiments in which both diffuse and unidirectional light are considered and in which the possibility of a low optimum intensity favoring attachment is borne in mind.

## Summary

1. Experiments were designed for the purpose of investigating the various anomalies reported in the response of barnacle larvae to black and to white surfaces. The apparatus consisted of panels of similar size but checkered with black and white squares of sides varying from eight inches to one inch.

2. Average attachment density over the entire panel increased slightly with diminishing size of squares. Attachment density to white squares increased with diminishing size of squares. Attachments to black squares did not vary continuously with size of squares. The distribution ratio between black and white decreased from over 3 on eight-inch squares to unity on 2-inch squares.

3. As an explanation of the results and of previously reported anomalies it is suggested that cyprids are stimulated to attachment by a low optimum light intensity of light, and that diffuse light rather than unidirectional light is the principal factor involved.

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# COMPARISON OF THE BINDING ABILITY OF HEMOCYANIN AND SERUM ALBUMIN FOR ORGANIC IONS

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## INTRODUCTION

The presence of hemocyanin in a dispersed form in the plasma of invertebrates suggests that this protein may act not only as a respiratory pigment but also as a transport vehicle for small ions. In mammalian plasma these functions are divided between two proteins, hemoglobin and serum albumin, respectively. The importance of the "vehicle function" of plasma albumin in facilitating or hindering the distribution of substances amongst various tissues and organs has been pointed out by Bennhold (1938) and by Davis (1946). No investigations have been reported on the interactions of hemocyanins with small ions, other than those involved in the respiratory function (Redfield, 1934; Dawson and Mallette, 1945).

A series of studies has been made, therefore, on the binding of several organic dye ions by the serum of the horseshoe crab, *Limulus polyphemus*. Parallel investigations for some anionic substances and bovine serum albumin have been reported previously (Klotz, 1946; Klotz and Walker, 1947). In the absence of quantitative data on the binding of cationic dyes by albumin, some experiments were carried out also with these substances and bovine serum albumin.

## METHODS AND MATERIALS

Quantitative measurements of the extent of binding by the proteins were made by the dialysis-equilibrium technique (Klotz, Walker and Pivan, 1946). A cellophane bag containing a measured quantity (10 cc.) of the serum, or of the solution of pure protein, was immersed in a large test tube containing a definite volume (20 cc.) of the solution of the small ion at a known concentration. The bag, impermeable to protein, was mounted on a glass frame from which a small bead was suspended by a Fiberglas thread into the protein solution. The test-tube was placed in a shaking device for twenty-four hours for the attainment of equilibrium. The bead in the protein solution served as a stirring device for the contents of the bag and thereby hastened materially the attainment of equilibrium. The bag was then removed and the external solution analyzed colorimetrically for the ion.

For each ion concentration, a control tube was prepared also, which differed from the primary tube only in that the former contained buffer rather than a protein solution or serum inside the bag. By this method it was possible to minimize any errors arising from binding of the ion by the cellophane membrane.

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[2] This investigation was supported in part by a grant from the Graduate School Research Fund of Northwestern University.

For each ion investigated, a preliminary series of experiments was run to ascertain the time necessary for the attainment of equilibrium. With the bead device described, a period of twenty-four hours was found to be more than sufficient in every case. A typical set of results for the cationic dye, chrysoidine, is illustrated by the data in Table I. The other substances showed similar behavior.

TABLE I

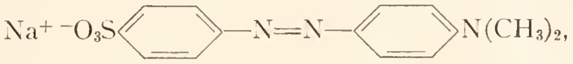
*Time for attainment of equilibrium in binding of chrysoidine*

| Time | Conc. of dye outside bag |
|---|---|
| 0.00 hours | $9.2 \times 10^{-5}$ moles/liter |
| 6.25 | 5.2 |
| 18.75 | 5.2 |
| 23.5 | 5.2 |

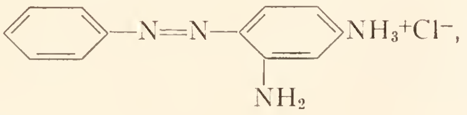
*Limulus* serum was obtained from a heart puncture of the animal.[3] Coagulation was permitted to occur at room temperature for about fifteen minutes. The serum obtained by subsequent filtration was diluted to four times its initial volume and stored in a refrigerator until used.

Bovine serum albumin was recrystallized material obtained from Armour and Company. Solutions of 0.2 and 2 per cent concentration were used in the binding experiments.

The methyl orange,



was a commercial sample of reagent grade.The chrysoidine,



was a commercial sample.

The dialysis experiments at pH 5.0 were carried out in 0.1 M acetate buffer solutions and those at pH 5.2 in 0.2 M phosphate. The pH values were measured with a Coleman glass electrode. Colorimetric analyses were carried out with a Klett-Summerson photoelectric colorimeter. All equilibria were attained in a water bath at 25 ± 2° C.

## RESULTS AND DISCUSSION

Data on the binding of methyl orange, a typical aromatic organic anion in its protein-binding properties (Klotz, 1946), by *Limulus* serum at a pH of 5.0 are summarized in Table II. It is evident that appreciable binding occurs with *Limulus* serum also, and that the extent of combination increases with the concentration of free dye. Relatively wide variations in the data are observed with this serum, however, probably because of differences between samples.

If one assumes that the serum contains about 4 per cent hemocyanin, then 10 cc. of the diluted solution within the bag contains approximately 0.1 gram of protein,

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[3] We are indebted to Miss Marguerite Webb for technical advice.

TABLE II

*Binding of methyl orange by* Limulus *serum, pH 5.0, acetate buffer*

| Conc. of free dye | Moles dye bound by hemocyanin in 10 cc. of diluted serum |
|---|---|
| $1.5 \times 10^{-5}$ M | $0.5 \times 10^{-7}$ |
| 2.7 | 1.6 |
| 3.8 | 2.1 |
| 3.8 | 2.1 |
| 5.3 | 3.3 |
| 5.6 | 2.7 |
| 5.8 | 1.8 |
| 10.0 | 6.9 |
| 10.8 | 3.9 |
| 10.8 | 6.3 |

and at a concentration of $1 \times 10^{-4}$ molar free dye, approximately $0.5 \times 10^{-5}$ mole of methyl orange would be bound by one gram of hemocyanin. This is about one-tenth the degree of binding observed with bovine albumin (Klotz, Walker and Pivan, 1946) where approximately $6 \times 10^{-5}$ mole of methyl orange is bound by one gram of protein at a free dye concentration of $1 \times 10^{-4}$ molar.

Several experiments were carried out with methyl orange and hemocyanin in a phosphate buffer at pH 5.2. The results obtained (Table III) indicate a slightly decreased degree of binding. However, the difference in the two pH's is too small to be attributed to changes in hydrogen-ion concentration for there may be small deviations due to differences in the ionic nature and strength of the buffer solutions.

TABLE III

*Binding of methyl orange by* Limulus *serum, pH 5.2, phosphate buffer*

| Conc. of free dye | Moles bound dye |
|---|---|
| $4.8 \times 10^{-5}$ M | $1.2 \times 10^{-7}$ |
| 6.1 | 1.6 |
| 8.6 | 2.4 |
| 11.9 | 3.6 |
| 17.6 | 9.3 |

In the case of albumin, there is strong evidence (Klotz and Walker, 1947) that electrostatic attraction between the organic anion and a positively-charged, quaternary nitrogen of one of the basic amino acids contributes a major portion of the binding energy. While the demonstration of a similar force in the hemocyanin complex requires further work on the effect of pH, it is, nevertheless, of interest to note that this protein like albumin is rich in the basic amino acids, histidine, lysine and arginine (Dawson and Mallette, 1945).

As an example of a cationic species, the dye chrysoidine was used at a pH of 5. No significant binding was observed, as is evident from the data in Table IV. The concentration of free cation was not reduced significantly by the presence of diluted *Limulus* serum inside the cellophane bag.

In the absence of comparable data for albumin, a series of binding experiments was carried out with chrysoidine and bovine serum albumin. The results obtained have been assembled in Table V. It is evident that chrysoidine is bound by albumin, though only about one-tenth as strongly as is methyl orange. If the same decrease in binding in going from the anion, methyl orange, to the cation, chrysoidine, holds in hemocyanin as is found with albumin, it would require more precise techniques than were available to detect the presence of a chrysoidine complex of hemocyanin.

Table IV

*Absence of binding of chrysoidine by* Limulus *serum, pH 5.0*

| Conc. of free dye in equilibrium with protein | Conc. of free dye in blank |
|---|---|
| $5.8 \times 10^{-5}$ M | $6.1 \times 10^{-5}$ M |
| 17.8 | 16.3 |
| 27.0 | 28.8 |
| 32.8 | 33.0 |
| 39.0 | 39.5 |
| 44.5 | 44.0 |

Table V

*Binding of chrysoidine by bovine serum albumin, pH 4.7*

| Conc. of free dye | $\frac{\text{Moles bound dye}}{\text{Moles total albumin}}$ |
|---|---|
| $1.1 \times 10^{-5}$ M | 0.0523 |
| 2.4 | 0.136 |
| 3.8 | 0.188 |
| 5.8 | 0.279 |
| 6.0 | 0.294 |
| 12.0 | 0.577 |
| 12.7 | 0.652 |
| 19.8 | 0.871 |
| 19.8 | 1.09 |
| 24.5 | 1.05 |
| 29.5 | 1.31 |
| 34.5 | 1.42 |

The binding data for chrysoidine with bovine albumin can also be expressed analytically by the equation

$$\frac{\text{moles protein}}{\text{moles bound dye}} = \frac{1}{k\,n}\,\frac{1}{(\text{free dye})} + \frac{1}{n}$$

where $k$ represents the intrinsic binding constant and $n$, the maximum number of chrysoidine ions which can be bound by a single albumin molecule. As has been shown previously (Klotz and Walker, 1947) this equation is derivable from the law of mass action for the ideal situation in multiple binding, i.e. one in which a bound ion exerts no electrostatic influence on successively bound ions. Thus within the precision of the present experiments, chrysoidine fulfills this ideal condition. The maximum number of sites, $n$, available to chrysoidine turns out to be 16. The intrinsic binding constant equals $3.1 \times 10^2$. From these values it follows (Klotz, Walker and Pivan, 1946) that the energy of binding of the first chrysoidine ion is about 5,000 calories/mole in comparison to 5,960 calories/mole for methyl orange.

Attempts were made also to obtain some information on the binding of methylene blue by albumin and hemocyanin, respectively. Unfortunately, the persistence of micelle formation by this dye even at exceedingly low concentrations made it difficult to interpret the data. Difficulties were also encountered in preliminary experiments on the binding of ferric iron, apparently because of oxidation of the cellophane membranes in the presence of this ion at the relatively high acidities (pH 3) which were necessary to avoid formation of colloidal iron oxide.

## CONCLUSIONS

The results obtained indicate that hemocyanin is capable of forming complexes with organic anions though the binding affinity is less than that observed with bovine albumin. Thus in addition to its function as a respiratory pigment, hemocyanin may be capable of acting as a vehicle in the transport of anions in the blood of invertebrates, as an agent for conserving desirable substances as the blood passes through the excretory organs and as a buffer against the effects of cytotoxic agents (Davis, 1946). In all of these actions, however, the present experiments indicate that its effectiveness is much less than that of serum albumin.

## SUMMARY

1. Hemocyanin forms complexes with organic anions, such as methyl orange, though with an affinity of about one-tenth that observed with the same dye and serum albumin.

2. No binding was observed between hemocyanin and the cationic dye, chrysoidine. Under comparable conditions bovine serum albumin combined with chrysoidine, thought quantitative calculations indicate an energy of binding about 1,000 calories less than that observed for albumin-methyl orange complexes.

3. The data indicate that hemocyanin acts not only as a respiratory pigment in the blood of invertebrates but also may serve to a limited extent in the distribution and conservation of organic ions among various organs and tissues.

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# DEVELOPMENTAL CHANGES IN THE VIABILITY OF SQUID EMBRYOS AFTER SUBJECTION TO CYANIDE

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## INTRODUCTION

As a consequence of data which have been accumulating for the past century, it is now well recognized that various animals typically considered as obligate aerobes are capable to a greater or less extent of withstanding exposure to oxygen lack (cf. the reviews by Slater, 1928, and v. Brand, 1946). This ability is not necessarily a static characteristic throughout the lifetime of an organism, however, for it has frequently been reported for example that the new born mammals of various species are definitely more resistant to asphyxia than are adults.

Much of the literature in recent times on the changing susceptibility of organisms to oxygen lack has dealt with the effects of anoxia upon such early embryonic processes as segmentation, gastrulation and neurulation (for bibliography see Needham, 1931). There are also, however, data which leave no doubt that various organisms exhibit changes in susceptibility to anoxia throughout much later phases of development. Amerling (1908) has noted that as the body length of Rana and Bufo tadpoles increases to 12 mm. there is a gradual decline in the resistance to oxygen lack. Similarly Burrows (1921) observed that whereas heart-fibroblasts from 4–5 day chick embryos could grow and contract in pure nitrogen for as long as 24 hours, those of 10–15 day embryos would exhibit no activity whatever under similar circumstances. More recently, the original observations on mammals have been confirmed and extended in an attempt to learn something of the chemical mechanism involved (Reiss, 1931; Selle, 1944; Himwich, Bernstein, Herrlich, Chesler and Fazekas, 1942; Glass, Snyder and Webster, 1944). It is now clear too that similar changes in ability to withstand asphyxia occur throughout the devolpment of the duck (Rostorfer and Rigdon, 1947).

Most of these researches dealing with anoxia, however, have been concerned either with very early or very late stages of development, or with adult animals. It was felt that it might be of some value to examine resistance to oxygen lack in a more systematic fashion during the period of development, which follows the establishment of the primary organ-systems. To do this, since the attainment of strict experimental anaerobiosis is a somewhat difficult matter, it was deemed desirable to render oxygen unavailable to the experimental animals by the use of a suitable respiratory inhibitor. Cyanide is such a compound for it is generally conceded to produce its primary inhibitory effects upon animal tissues by a depression of oxidative metabolism. Inasmuch as it interferes with the intracellular transport of oxygen, it imposes essentially the same conditions upon the organism as does oxygen lack.

A series of studies has, therefore, been undertaken to examine the resistance of several species of Metazoa to the action of cyanide at various stages throughout development. The present paper deals with the effect of N/1000 CN upon the general viability of the squid, *Loligo pealei,* from about the fourth day of development to maturity. This concentration of cyanide causes in most tissues a maximal inhibition of oxygen consumption (Dixon and Elliot, 1929). To provide correlative data, a few measurements were made at various stages of the normal oxygen consumption and the effect of cyanide on it, and of the normal carbon dioxide production. In a few instances organisms were subjected to relatively high concentrations of carbon dioxide in order to determine whether or not the respiratory pigment of the blood was necessary for life at the developmental stage in question.

It was considered that experiments of this kind on the squid would be of particular interest in view of the very pronounced susceptibility of the adult of this species to anoxia.

## Material and Methods

Mature squid (*L. pealei*) of both sexes were obtained fresh from the nets or live-boxes of the Collecting Department of the Marine Biological Laboratory and were placed in a large aquarium of running seawater containing anchored algae. The marine minnow, *Fundulus heteroclitus,* was used as food and the tank was covered and kept dark until eggs were deposited. In this way the animals remained healthy, eggs in abundance were obtained in one to two days, and the time at which the eggs were deposited could be determined to within a few hours.

The eggs from each female were kept separately in gently running seawater. After the first day or two of development the strings were separated from one another, freed of the two outermost gelatinous envelopes, and cut into ½–1-inch lengths to permit the same rate of development in all embryos.

Samples of normal embryos were fixed in saline formalin (Microtomist's Vade-Mecum, p. 458) at the time of each experiment. Their stage of development was determined by comparison with the figures of Naef (1928) for *Loligo vulgaris* rather than with those published earlier for *L. pealei* because Naef's monograph is more commonly available. This procedure seems permissible since, according to Naef, there is little or no evidence that the species are distinct.

The time scale of development which will be employed in presenting the observations allows only two-thirds of a day per stage instead of one day as in *L. vulgaris* (Naef, 1928). This presumes a total of 12 days from oviposition to hatching which is the usual period cited at Woods Hole for the American squid (Grave, unpublished). The actual numbers of days required for five typical batches of eggs to hatch along with the corresponding temperatures are given in Table I.

Table I

| Laid | Hatched | Days required for development | Extremes of temperature for the period of development |
|---|---|---|---|
| June 8 | June 30 | 22 | 14.2–17.2 |
| June 12 | July 5 | 23 | 15.6–18.6 |
| June 13 | June 26 | 13 | 15.8–16.9 |
| July 19 | Aug. 3–5 | 15–17 | 17.8–21.1 |
| July 21 | Aug. 4 | 14 | 17.8–21.1 |

The concentration of the Stock solution of cyanide was standardized at intervals, and prior to an experiment the pH of the N/1000 solution of cyanide in seawater to which the organisms were to be exposed was, if necessary, adjusted to the pH of seawater.

The respiration experiments were performed by the direct method of Warburg (Dixon, 1943). Twenty or thirty embryos were placed in each respirometer vessel. The approximate volume of the animals was previously determined by placing them in a small graduated cylinder and adding a measured quantity of seawater.

In determining the effect of cyanide on the rate of oxygen consumption, each experiment usually included two controls and two or more vessels to which was added sufficient N/100 cyanide at the pH of seawater to bring the cyanide concentration to approximately N/1000. In some experiments the inhibitor was added at the beginning. In others the vessels were removed from the manometers after a preliminary determination of the normal rate of oxygen consumption and the inhibitor then added. With cyanide present the insets contained the mixture of sodium cyanide and sodium hydroxide suggested by Krebs, 1935.[1] The temperature in the initial experiments varied from one experiment to another over the range 21–25° C. Later it was maintained constant at 22° C. The duration of the respiration experiments varied from 3½ to 8½ hours.

The rate of oxygen consumption and carbon dioxide production are expressed on the basis of number of embryos rather than weight of respiring tissue because of the presence at most stages of development of the jelly and of a large amount of inert yolk.

Solutions of carbon dioxide were made up immediately before use by adding various quantities of seawater previously saturated with carbon dioxide to about 8 liters of fully aerated seawater. The animals were quickly introduced, a protecting screen was submerged over them and the surface of the water was then covered with a thick layer of paraffin oil. Where both embryos and adults were used together, the former were confined in a piece of glass tubing of approximately 1½ in. bore, the ends of which were closed by a single layer of cheese cloth.

The authors are indebted to Virginia S. Black for determinations of the carbon dioxide partial pressures by the method of Krogh (1908).

## RESULTS

### *A. Viability after exposure to cyanide*

When squid embryos are placed in dilute solutions of cyanide, certain characteristic symptoms very soon make their appearance. If the embryo normally shows rotation within the jelly and pulsation of the yolk sac (which is first seen in Stage XIV), these activities become slower and after approximately 10 minutes, cease. The hearts however continue to beat in cyanide though at a gradually declining rate for as long as two hours. The "oxidative reserve" available to the heart is clearly of greater magnitude than that maintaining the pulsation of the yolk sac.

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[1] According to the data published by Robbie (*Jour. Cell. and Comp. Physiol.*, vol. 27, pp. 181, 1946), the cyanide concentration surrounding the organisms in the present experiments probably increased with time due to the uptake of carbon dioxide by the cyanide-hydroxide mixture in the inset. Since no appreciable change of inhibition occurred with time even the initial concentration of .001 N must have produced a maximal effect.

In embryos near hatching, and in larvae or adult squid, immersion in cyanide almost immediately produces acute respiratory distress, as indicated by greatly increased amplitude of mantle and siphon movements. Soon afterwards these movements cease. In addition, the chromatophoral system undergoes a characteristic cycle whereby the animals flush at first to a deep red or purple, then blanch permanently to a bluish-white color.

The criteria used for recovery were, in general, the reverse of the symptoms outlined above:

(a) In young embryos rotation within the egg capsule, pulsation of the yolk sac, and continuation of external development.

(b) In young hatching larvae and adults, resumption of normal body color (as opposed to the blanched condition), recovery of respiratory movements, and the ability to swim freely.

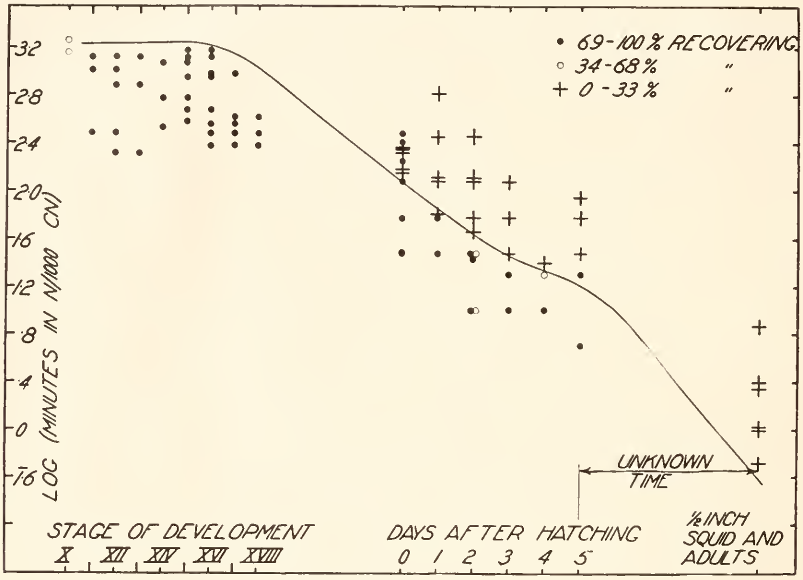

Figure 1. The number of organisms which recovered after subjection to cyanide for the periods indicated. Each entry represents a single experiment in which, as a rule, at least ten organisms were used.

It should be mentioned here that for the purposes of this paper, observations on "recovery" do not deal with the effect of cyanide upon subsequent morphology. Subjection to the inhibitor for long periods of time (i.e., 10 hours) frequently appears to have a subsequent teratological effect upon squid embryos which becomes evident at the time of formation of mantle and siphon. There may also be distortion of the yolk sac, though the embryo apparently continues to derive nourishment from it.

In a general way recovery is most rapid in the embryo, less so in the larvae, and least rapid in the adult. Squid younger than two days post-hatching show remarkable "reversibility" when cyanide is replaced by normal seawater. In one case, mantle pulsations began within 8 minutes, and at this time the rate of the heart was very appreciably accelerated. One group of embryos which had been in cyanide for 17 hours recovered after two hours in seawater. Another group, subjected to cyanide for over 11 hours, resumed rotation and yolk-sac movements after only 5 minutes in seawater.

In Figure 1 are shown the viabilities of squid of various stages after immersion in cyanide for varying lengths of time. Each entry on this chart represents a single experiment in which, as a rule, at least ten organisms were used. If no more than one-third of the organisms subjected to cyanide survived, the entry for this experiment is shown as a cross; if between one- and two-thirds survived, the entry is a circle; and if between two-thirds and all of the organisms were viable, the entry is made as a point. Over eighty experiments are represented involving ten separate clutches of eggs.

It is apparent from this figure that embryonic squid are extremely resistant to cyanide, since at Stage X all survive 20 hours exposure to the N/1000 solution. In fact, according to a number of observations made by Barnes (1939, personal communication), segmenting eggs and embryos younger than Stage X are still more resistant. It must be pointed out, however, that the development subsequent to these long exposures is accompanied by a certain amount of deformity, so that perfectly normal ontogeny under these circumstances is unusual.

As development proceeds, the squid becomes less and less able to withstand the influence of cyanide, a fact which undoubtedly reflects a declining capacity for tolerating oxygen lack. A day or two after hatching death results from an exposure to cyanide of about one hour; adults however cannot survive an immersion of one minute. In Figure 1 a line has been drawn to indicate in an approximate fashion the time course of the change in viability with age. From the data shown in Figure 1 the longest exposure to cyanide which can be tolerated without death occurring has been estimated for the different developmental stages. These threshold durations are given in Table II.

TABLE II

| Stage of development | Threshold-duration in N/1000 NaCN |
|---|---|
| X–XVI | 20 hours |
| XVII | 17 hours |
| XVIII | 7 hours |
| Hatching | ca. 2 hours |
| 1-day larva | ca. 1 hour |
| 2-day larva | ca. $\frac{1}{2}$ hour |
| 4-day larva | ca. $\frac{1}{4}$ hour |
| Half-inch squid and adults | 1 minute |

It was noted in the experiments with 4-day larvae that the hearts continued to function even after the organism had been exposed to cyanide for an hour, an exposure which is, of course, fatal. A similar relative insensitivity of the heart to oxygen lack was reported by Redfield and Goodkind (1929). They found in adult squid asphyxiated by raising the carbon dioxide content of the water respired, that an hour after respiratory movements had ceased the heart was still beating.

Clearly, death from oxygen lack is not due in the first instance to any functional impairment of the circulatory system.

In contrast both the respiratory and locomotory movements of the mantle and siphon, which disappear very quickly in the adult upon exposure to cyanide, also fail to return to any appreciable extent after poisoning by cyanide. Thus not only does cyanide, and presumably oxygen lack, very quickly interfere with the function of the central nervous system, but these effects show less reversibility than do those of, for example, the circulatory system. This may perhaps be taken to indicate that the essential basis of the sensitivity to anoxia resides in the nervous system.

### *B. Effect of carbon dioxide*

Redfield and Goodkind (1929) have shown that in the adult squid relatively low partial pressures of carbon dioxide prevent the haemocyanin of the blood from exercising its normal function in oxygen transport. Subjection of adult squid to partial pressures of carbon dioxide thus creates asphyxial conditions and brings about death from oxygen want. The respiratory function of the blood is quite completely eliminated, according to these investigators, by carbon dioxide partial pressures of 30–50 mm. Hg. We have compared therefore, in a few experiments, the reactions of adult and immature squid to carbon dioxide partial pressures in that range. The exact conditions employed and the observations which were made are summarized in Table III. These data show that at least until two days after hatching *L. pealei* is quite unaffected by a 20-minute exposure to a carbon dioxide pressure as high as 45 mm. Hg. It is also very apparent that such an exposure is fatal to adults. However, larvae 1–2 days old fail to withstand a 48 mm. partial pressure of carbon dioxide for 30 minutes. Even this treatment does not affect younger (Stage XVIII) larvae. Confirming Redfield and Goodkind we find that adults show no recovery whatever after an exposure to a 26 mm. partial pressure of carbon dioxide for 15 minutes.

Table III

| Stage | Temp. °C. | Av. p $CO_2$ mm. | Duration of exposure minutes | Result |
|---|---|---|---|---|
| XIV | 24.3 | 40.0 | 30 | Complete recovery |
| XVII–XVIII | 23.3 | 48.3 | 30 | Complete recovery |
| Hatching | 24 | 37.1 | 31 | 65% recovery |
| Hatching | 23.3 | 48.3 | 30 | 11% recovery |
| Hatched 1 day | 23.4 | 45.0 | 19 | Complete recovery |
| Hatched 1–2 days | 23.4 | 45.0 | 19 | Complete recovery |
| Hatched 1–2 days | 23.3 | 48.3 | 30 | No recovery |
| Hatched 2 days | 23.4 | 45.0 | 19 | Complete recovery |
| Adult (2) | 20–25 | 26.2 | 16 | No recovery |
| Adult (1) | 24 | 37.1 | 12½ | No recovery |
| Adult (2) | 21.5 | 40.0 | 28 | No recovery |
| Adult (1) | 23.4 | 45.0 | 19 | No recovery |
| Adult (1) | 23.3 | 48.3 | 37 | No recovery |

It is apparent from these data that resistance to carbon dioxide roughly parallels sensitivity to cyanide, and it seems probable therefore that carbon dioxide is in fact producing asphyxia just as does cyanide. Furthermore it must be concluded that in all the stages examined, the normal carriage of oxygen to the tissues of the organism requires the intervention of haemocyanin as a carrier of oxygen. The respiratory function of the blood pigment thus appears to be well established at a time when resistance to cyanide (asphyxia) is still relatively high.

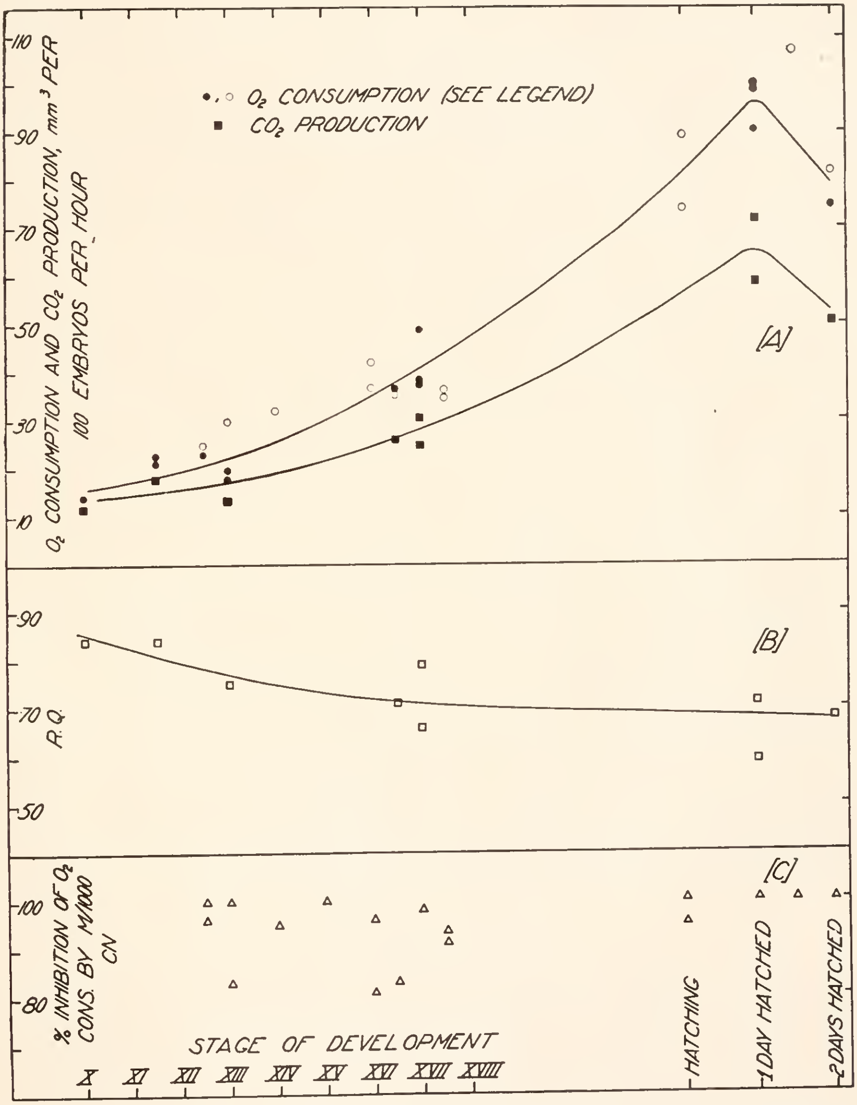

FIGURE 2. The rates of oxygen consumption and of carbon dioxide production, together with the respiratory quotient and the cyanide sensitivity, at different stages of development.

### *C. Respiration*

The results of a series of experiments in which the normal rate of oxygen consumption of squid was determined at various stages of development are represented graphically in Figure 2a and b. Over the period examined a four- to eight-fold increase in the rate of oxygen consumption occurred. It is apparent from Figure 1 and Table II that over the same period the resistance to CN changed over forty-fold. While in general, then, the rate of oxygen consumption rises as the tolerance to asphyxia decreases, the latter actually changes 5–10 times as much as the former.

The drop in respiratory rate suggested shortly after hatching (Figure 2a), if real, may be associated with the rapid utilization of the remaining yolk as a result of increased activity of the organism and the failure to obtain natural food. Very little is known of the preferred food of squid larvae (Portmann and Bidder, 1928). In spite of the presence of abundant plankton, in our experience they survived only 4–5 days after hatching, dying apparently from starvation.

When squid embryos were exposed to cyanide in the respirometers, the respiration was observed to decline almost instantly (within a few minutes), indicating the immediate penetration of cyanide into the eggs. The rates of oxygen consumption in cyanide at the various stages are indicated in Figure 2c, from which figure it is evident that, in general, a high degree of inhibition (approximately 95 per cent) persists throughout development. It seems doubtful whether any significance can be attached to the apparent drop in sensitivity at Stages XVI–XVII. As far as the utilization of oxygen is concerned, therefore, there appears to be no evidence of a change in relative cyanide-sensitivity with age. It may be concluded that viability in this organism does not appear to be associated in any way with the possession of a cyanide-stable oxygen consumption.

Upon nine occasions, distributed over various stages of development, the normal rate of carbon dioxide production was also determined. From measurements of normal oxygen consumption made at the same time, the respective R.Q.'s were calculated and will be found in Figure 2b. The values show a slight tendency to rise during the early stages but there are no large fluctuations. This indicates that, as might be expected, the rate of carbon dioxide production rises throughout development just as does the rate of oxygen consumption. No marked changes in R.Q. are evident and there are certainly no changes which might be associated with the observed changes in viability on exposure to cyanide.

## DISCUSSION

The fact that the young of certain species are much better able to tolerate asphyxia than are the adults, has been known for a very long time as Johlin (1942) has recently emphasized. The newer work, which has already been referred to deals almost entirely with several of the common mammals. It might leave the impression that the decreasing tolerance of asphyxia seen in these during the few weeks following birth is a peculiarity of higher forms. Actually, this is apparently not the case, for the present work on squid indicates that in this species as well, young embryos typically survive exposures to asphyxia which are fatal to older embryos and to adults. Furthermore, the change in viability with development does not appear to be confined to any particular phase of that process, but instead takes place gradually from the earliest stages examined. It will be shown elsewhere that an exactly similar change in viability with development occurs in several species of fish, and in frogs, so that in all probability it is quite a general phenomenon.

It is of course altogether likely that energy normally supplied by aerobic processes is replaced under asphyxial conditions by energy from anaerobic processes. The observations of Himwich, Bernstein, Herrlich, Chesler and Fazekas (1941) are particularly suggestive in this connection. These investigators report that the survival of newborn rats placed in an atmosphere of nitrogen is significantly shortened if the animals are also treated with iodoacetate. One might conclude that viability depends upon some particular cells and that the anaerobic capabilities of these become less as development proceeds. A priori however, it is just as logical to suppose that early in development viability depends on cells whose anaerobic capabilities are great, while later it comes to depend on the functioning of some more recently differentiated cells whose anaerobic capabilities are very much less. Supporting this latter conclusion are the facts (1) that the lethal effects of asphyxia are almost certainly exerted within the nervous system (Kabat and Schadewald, 1941; and Weinberger, Gibbon and Gibbon, 1940); (2) that the cells of the nervous system differ in their resistance to asphyxia (Kabat and Schadewald, 1941; Weinberger, Gibbon and Gibbon, 1940; van Harreveld, 1944); and (3) that in the mammal, at least, differentiation in the nervous system is taking place during those post partum stages in which the ability to survive asphyxia shows marked changes (Himwich and Fazekas, 1941).

## Summary and Conclusions

1. The exposure to 0.001 N cyanide, which is fatal, gradually shortens from more than 20 hours in early embryos to 15 minutes in organisms examined four days after hatching. It is less than one minute in adults.
2. Cyanide brings about at least a 95 per cent inhibition of the rate of oxygen consumption of squid embryos at all stages examined. This inhibition develops immediately upon the addition of cyanide to the organisms.
3. A few data were obtained relating to the ability of squid embryos and adults to withstand increased partial pressures of carbon dioxide. It was found that this ability decreases as the embryos become older. The effect of the carbon dioxide is almost certainly to make the haemocyanin of the blood incapable of carrying oxygen. It is concluded that at all stages examined the normal rate of oxygen consumption requires the participation of the respiratory pigment of the circulation.
4. It may be inferred from these data on the effects of cyanide and of carbon dioxide that embryos are much more resistant to lack of oxygen than are adults. This general situation has been reported for certain other organisms. In the case of squid it is now evident that this change in resistance to asphyxia is a gradual process which is not related to any particular phase of development.
5. It is pointed out that it is undoubtedly damage to the central nervous system which is the immediate cause of death from oxygen lack, and that the site of the change in sensitivity to oxygen lack must therefore be sought in the central nervous system.

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# CARBONIC ANHYDRASE IN MOLLUSCS[1]

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The catalytic action of carbonic anhydrase in the hydration of carbon dioxide suggests the possible role of this enzyme in the deposition of carbonate in shell (Meldrum and Roughton, 1933). This relationship has received consideration with respect to shell formation in birds in which it has been found that the shell-forming tissues contain carbonic anhydrase and inhibitors of that enzyme cause deficient shell formation (Common, 1941; Benesch, Barron and Mawson, 1944; Gutawska and Pozzani, 1945).

In molluscs the $CaCO_3$ of the shell is presumably formed first in mantle tissue with at least a portion of the carbonate having its origin in metabolic carbon dioxide (Robertson, 1941). The influence of carbonic anhydrase on the conversion of carbon dioxide to carbonate in molluscs has not been studied experimentally, though the enzyme has been reported in mantle tissue of a few pelecypods (Florkin and de Marchin, 1941; Maetz, 1946) as well as in other tissues of molluscs (van Goor, 1937; Sobotka and Kann, 1941). As an approach to this problem the carbonic anhydrase content of mantle tissues has been examined in 20 species of molluscs.

## METHODS

Tissues were blotted, weighed and ground thoroughly with a closely fitting mortar and pestle. Distilled water equivalent to 50 times the tissue weight was added and the suspension extracted 12 to 24 hours below 5° C. (Ferguson et al., 1937). Body fluids underwent similar storage without dilution. Extracted suspensions were agitated vigorously and pipetted directly without previous centrifugation. In one set of experiments tissues were blotted, placed in vials and kept frozen for several days. They were then extracted as previously described. Enzyme values for these tissues were similar to those for freshly extracted material.

The hydration of $CO_2$ in mantle tissue undoubtedly occurs in the presence of both carbonate and bicarbonate; and the colorimetric method (Brinkman, 1933; Leiner, 1938) used in the present study in which $CO_2$ is added to a carbonate-bicarbonate solution is appropriate for the estimation of carbonic anhydrase activity in this tissue. However, the values so obtained will include the specific action of the buffer and indicator on the enzyme (Roughton and Booth, 1946). The endpoint was determined by means of a comparison tube containing phenol red buffered at pH 7.35, a value suitable both from the standpoint of rapidity of color change and rate of enzyme action. The activity of tissue extracts was measured at a final concentration of 1 : 500 and body fluids at 1 : 10. Two types of controls were used. In one the time required for reading the endpoint was determined with distilled water substituted for the extract, and in the other a portion of the extract was heated in

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[1] Aided by a grant from the Duke University Research Council.

TABLE I

| Species | Ratio I: $\frac{\text{No extract}}{\text{Extract}}$ | Ratio II: $\frac{\text{Heated extract}}{\text{Extract}}$ |
|---|---|---|
| *Pelecypoda:* | | |
| *Atrina rigida* | | |
| mantle edge | 0.8; 1.0; 0.9; 1.0; 1.0 | 1.1; 1.1; — — — |
| mantle without edge | 0.7; 1.0; 0.9; 1.0; 0.9 | 1.0; 1.1; — — — |
| pericardial fluid | 0.6; 0.9; 0.7; 0.6; 0.9* | 1.2; 1.1; — — — |
| gill | 2.8; —; 2.2; 3.4; — | 4.2; —; — — — |
| adductor muscle | —; 0.8; 1.5; 1.2; — | —; 0.6; — — — |
| *Venus mercenaria* | | |
| mantle edge | 2.6; 4.8; 2.5; | 3.4; 7.2; |
| mantle without edge | 3.3; —; 2.6; 4.1 | 4.8; — |
| mantle cavity fluid | 0.8; 1.4; 2.0; | 1.5; 3.5; |
| *Ostrea virginica* | | |
| whole mantle | 4.9; 1.5; 1.8; 3.6; | 7.2; 2.2 |
| general body fluid | 7.1; 2.2; —; —; | |
| gill | 2.8; —; 5.5; —; | 3.7; |
| adductor muscle | —; —; 1.0; —; | |
| *Macrocallista nimbosa* | | |
| mantle edge | 1.2; 1.0; 1.5; 1.9 | 2.0; 1.4; |
| mantle without edge | 1.8; 2.1; 1.6; | 3.0; 3.1; |
| mantle cavity fluid | 0.6; | 1.5; |
| gill | 5.0 | |
| adductor muscle | 1.2; | |
| *Dosinia discus* | | |
| mantle edge | 1.1; 1.9; 2.1; | 1.4; |
| mantle without edge | 2.0; 2.3; 2.5; | 3.1; |
| body fluid | 1.5; 14.0*; 1.1*; | 3.9; |
| adductor muscle | 10.0; 6.4 | —; —; —; 4.0 |
| *Elliptio complanatus* | | |
| mantle edge | 2.5; 2.0; | 2.6; 2.1; |
| mantle without edge | 2.8; 2.5; | 3.0; 2.6; |
| mantle cavity fluid | 4.7; 1.7; | 5.1; 2.0; |
| adductor muscle | 3.0; 3.7; | 2.4; 4.1; |
| *Tagelus gibbus* | | |
| mantle edge | 2.7; 2.1; | |
| mantle without edge | 3.4; 4.2; | |
| mantle cavity fluid | 2.7; 1.6; | |
| gill | 5.0; | |
| *Pecten irradians* | | |
| whole mantle | 2.5; —; 2.0 | |
| mantle edge | 5.2; 2.4; — | |
| pericardial fluid | —; —; 1.4 | |
| *Divaricella quadrisulcata* | | |
| whole mantle* | 3.2 | |
| *Ensis directis* | | |
| mantle edge | 3.4 | |
| mantle without edge | 8.1 | |
| *Modiolus tulipus* | | |
| whole mantle | 2.0 | |
| *Cardium muricatum* | | |
| whole mantle | 4.3 | |

TABLE I—*Continued*

| Species | Ratio I: $\frac{\text{No extract}}{\text{Extract}}$ | Ratio II: $\frac{\text{Heated extract}}{\text{Extract}}$ |
|---|---|---|
| *Gastropoda:* | | |
| *Busycon carica* | | |
| whole mantle | 1.2; 0.8; 1.6; 1.1; 3.0; 2.1 | 1.7 |
| blood | 0.6; —; 0.8; 0.6; | 1.9 |
| gill | 1.8; | |
| *Viviparus japonicus* | | |
| whole mantle | 1.4; 1.6 | 1.9; 2.2 |
| mantle cavity fluid | 0.6; 0.9 | —; 1.0 |
| *Crepidula fornicata* | | |
| whole mantle | 0.4; 0.6; 1.2 | |
| mantle cavity fluid | 0.9; | |
| *Fasciolaria distans* | | |
| whole mantle | 2.4; 5.1; | |
| blood | 2.3; | |
| *Anomia simplex* | | |
| whole mantle* | 3.6 | |
| *Polynices duplicata* | | |
| whole mantle | 2.7 | |
| *Sinum perspectivum* | | |
| whole mantle | 2.8 | |
| *Diodora alternata* | | |
| whole mantle | 2.0 | |

* Sample composite of tissues from several individuals.

boiling water for 5 minutes. The activity is expressed as the ratio of the time of reaction in the absence and presence of the extract and also as the ratio of time of reaction for heated and unheated extracts. Duplicate determinations were made in a large proportion of the cases and the average taken.

We are indebted to Mr. W. H. Sutcliffe, Jr., for aid in the collection of material and to Dr. C. G. Bookhout and Dr. H. van der Schalie for its identification.

## RESULTS

Each figure in Table I represents the relative catalytic action of a single sample. Tissues from a particular individual are grouped in columns. In those cases in which both ratios were determined for the tissue the figures stand in corresponding positions under the two ratio headings.

It is evident that most of the tissues examined catalyzed the hydration of $CO_2$. Considered from the standpoint of Ratio I the outstanding exceptions are certain tissues of *Atrina rigida, Macrocallista nimbosa, Busycon carica, Crepidula fornicata,* and *Viviparus japonicus.* However, several of these instances of the absence of catalysis are only apparent as shown by comparing heated and unheated extracts (Ratio II). Of the individuals examined, carbonic anhydrase activity under the conditions of measurement is negligible or absent only in the mantle and pericardial fluid of *Atrina rigida,* the mantle-shell cavity fluid of *Viviparus japonicus,* and perhaps also in the mantle and mantle-shell cavity fluid of *Crepidula fornicata.*

The higher values for Ratio II result from the slowing of the uncatalyzed reaction by the heated extract and are probably due to the presence of carbonate and other substances.[2] Figures given by Maetz (1946) show an analogous effect. Because of this effect the values (Ratio I) of all tissues for which data on heated extracts are not available may be considered minimal except for muscle. The four adductor muscles examined showed an opposite effect and one which would be expected with acid production.

Carbonic anhydrase activity was demonstrated in the body fluids of nearly all forms studied and in the blood of two species of gastropods. Florkin (1935) and van Goor (1937) investigated blood of a large number of species and found no evidence of the enzyme. This may have been the result of the high dilutions employed.

## Discussion

The presence of carbonic anhydrase in the mantle tissues of most of the molluscs examined indicates the possibility that this enzyme plays a part in the formation of carbonate. Both the mantle edge and the body of the mantle contain the enzyme and both are concerned with the deposition of carbonate (Robertson, 1941). Of course the presence of the enzyme cannot be taken as evidence that normal shell formation is dependent upon its activity, and its presence in other tissues suggests additional functions. The negligible activity in *Atrina rigida* and *Crepidula fornicata* suggests that certain forms may, in fact, lay down shell in its absence. In this connection a study of the rate of shell formation in the presence of inhibitors would be suggestive, provided the inhibitors did not interfere with the growth of other structures. The catalytic action of carbonic anhydrase may be expected to assume increased importance as the temperature is decreased, especially in forms such as the oyster in which shell formation proceeds during hibernation at temperatures below 4° C. (Galtsoff, 1934).

## Summary

Carbonic anhydrase has been demonstrated in the mantle tissues and body fluids of most of 12 species of pelecypods and 8 species of gastropods. Its presence suggests the possibility of importance in shell formation in most species but negligible activity in some indicates they may deposit shell in its absence.

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[2] This effect is probably complex and may involve: (1) a change in buffer action, (2) the effect of specific substances on the enzyme, (3) an alteration of the initial pH, and (4) modification of the properties of the extract in a manner other than in destruction of the enzyme.

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# PHYSIOLOGY OF INSECT DIAPAUSE. III. THE PROTHORACIC GLANDS IN THE CECROPIA SILKWORM, WITH SPECIAL REFERENCE TO THEIR SIGNIFICANCE IN EMBRYONIC AND POSTEMBRYONIC DEVELOPMENT

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In recent years it has become increasingly evident that insect metamorphosis is under the control of an array of factors generally presumed to be hormonal in character. Interest has heretofore centered around the endocrine functions of the brain, the corpora cardiaca, and the corpora allata, including the "ring gland" of Diptera. Since these organs are usually situated in the head, it has not been easy to account for the fact that a thoracic center exercises important control over the embryonic and postembryonic development of many species (Richards and Miller, 1937; Richards, 1937).

The existence of a thoracic "differentiation center" seems, indeed, to be a distinctive feature of the embryology of all insects (Schnetter, 1934). As will become evident subsequently, the position of this embryonic center is of special interest. According to Richards and Miller (page 35), it "extends from the second gnathal (maxillary) segment to the second thoracic segment with its midpoint in the anterior half of the presumptive prothorax."

In the case of postembryonic development the evidence in favor of the existence of a thoracic "differentiation center" has been especially prominent in studies of the Lepidoptera. Here the thorax is believed to preside over moulting (Fukuda, 1940b), pupation (Bodenstein, 1938; Bounhiol, 1938; Fukuda, 1940a), and adult development (Hachlow, 1931; Bounhiol, 1938; Fukuda, 1941; Williams, 1947). The thorax has not been implicated, to date, in the postembryonic development of the Diptera, Orthoptera, or Hemiptera, but according to Geigy and Ochsé (1940) the thorax or first abdominal segment controls pupation in the megalopteran, *Sialis*.

These observations take on renewed interest as a result of Fukuda's studies of the commercial silkworm, *Bombyx mori*, where pupation and adult formation were found to be dependent on the function of a previously overlooked organ, the "prothoracic glands." For the first time the identity of a thoracic differentiation center was thereby established. The prothoracic glands therefore merit special attention, for homologous structures may be widely distributed among insects and conceivably act as the thoracic center that is known to control embryonic and, in certain species, postembryonic development. For this reason the existing literature pertaining to the prothoracic glands will be briefly reviewed.

Prothoracic glands were first described by Toyama (1902) in his study of the embryology of *Bombyx mori*. They arise early in embryonic development as

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[1] The assistance of the Lalor Foundation of Wilmington, Delaware, is gratefully acknowledged.

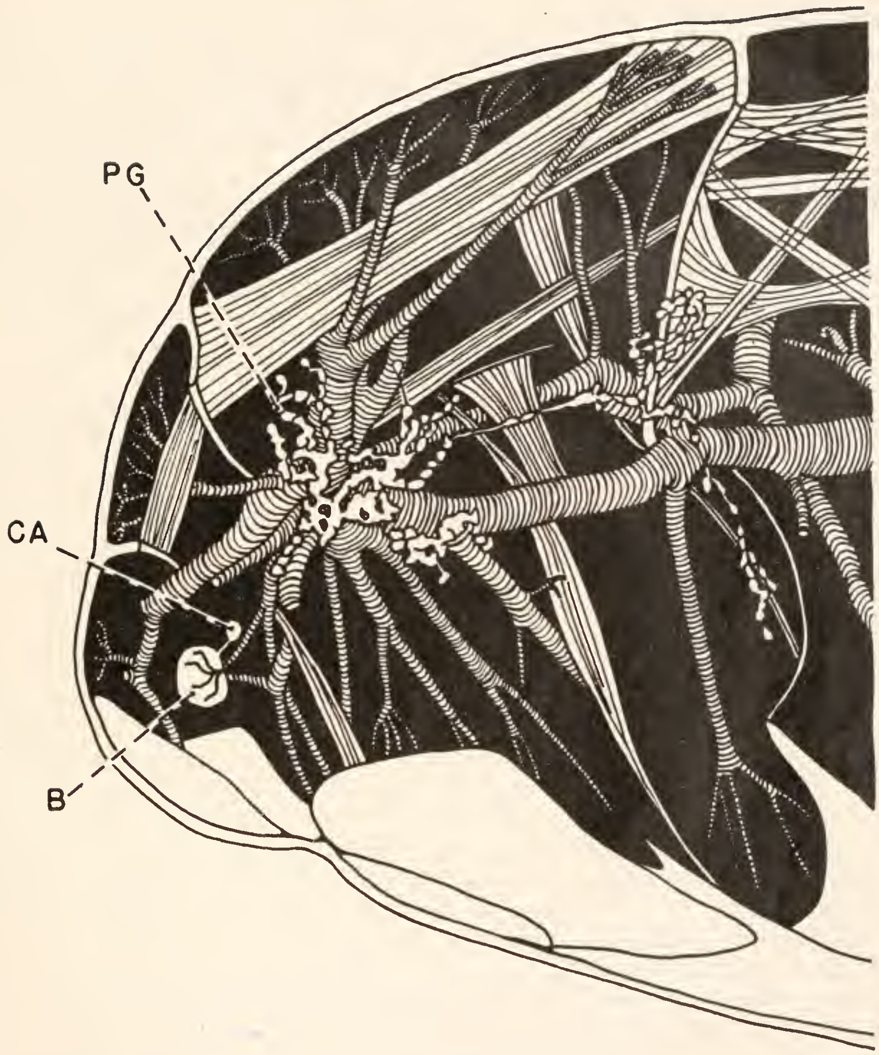


FIGURE 1. The prothoracic gland in the right half of the thorax of a diapausing Cecropia pupa. Fat body and numerous other tissues have been cleared away: B, Brain; CA, Corpus allatum and Corpus cardiacum; PG, Prothoracic glands.

epithelial invaginations of the lateral part of the second maxillary segment, from which they extend posteriorly into the prothorax. This location may, to advantage, be compared with that of the "differentiation center" in embryonic insects, described above.

Although Toyama's discovery has been generally overlooked, it is to his credit that he recognized the prothoracic glands not only as being hitherto unknown, but also as being glandular organs. They were named "hypostigmatic glands," an unfortunate designation, since the term had previously been applied to wholly different tissues in the Lepidoptera (Verson and Bisson, 1891). Twenty-eight years elapsed before the glands were next mentioned in the literature. Ke (1930) then named the organs "prothoracic glands" and described certain variations in their branching. In a paper to be published in the near future, Lee (1948) will present a morphological study of the prothoracic glands in lepidopterous larvae, with special reference to their innervation.

Physiological function for the prothoracic glands was first discovered by Fukuda (1940a, 1940b, 1941) in a series of studies reviewed elsewhere (Williams, 1948). Most recently, the function of the prothoracic glands has been considered in relation to adult development of several species of silkworms (Williams, 1947, 1948).

## Morphology and Histology

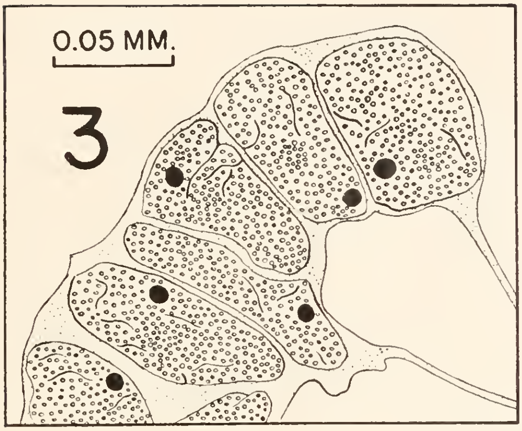
In my experience the prothoracic glands can be demonstrated most satisfactorily by gross dissections of fresh material submerged in insect Ringer's solution. Even under these circumstances the organs are not easy to find on account of their transparency. This difficulty may be minimized by vitally staining the preparation with methylene blue, a treatment that stains the prothoracic glands more or less intensely without impairing their viability in physiological experiments.

The following description is based on numerous dissections of the Cecropia silkworm and especially of the diapausing pupa of this species:

As shown in Figure 1, the prothoracic glands are bilaterally placed in the pro- and mesothorax. Each gland consists of a main mass and a number of branches. The main mass lies immediately internal to the large tracheae at the level of the prothoracic spiracle. Branches radiate from this main mass, as indicated in Figure 1. The branch extending in the direction of the brain is short and compact, but the other branches consist, for the most part, of fusiform or stellate cells strung together on tenuous, intercellular bridges. In the region of the mesothoracic spiracle the cells of the gland show a second concentration. All of these branches are laterally placed in the insect and are covered and partially entwined with fat-body. The gland is richly supplied with nerves and tracheoles.

The microscopic structure of the gland is indicated in Figures 2 and 3. Little internal structure is visible in the living gland (Fig. 2) and greater detail is not obtained after fixation and staining with most agents. In my experience, best results are obtained by fixation in Helly's solution and staining with haematoxylin. As shown in Figure 3, the gland is then found to consist of enormous, complexly folded nuclei surrounded by a scanty, cytoplasmic syncytium. Each nucleus contains a single, prominent nucleolus.

Although the above description applies, specifically, to the diapausing pupa, the prothoracic glands show essentially the same arrangement in the later larval instars. With the onset of adult development, dissection becomes very difficult, but the glands are still present and seem to assume a more racemose configuration in the region of the prothoracic spiracles. I have not succeeded in demonstrating the organs in the adult and believe them to be absent. It is probable that a description of the glands in young larvae, in developing pupae, and in the adult (?) must await reconstruction from serial sections.



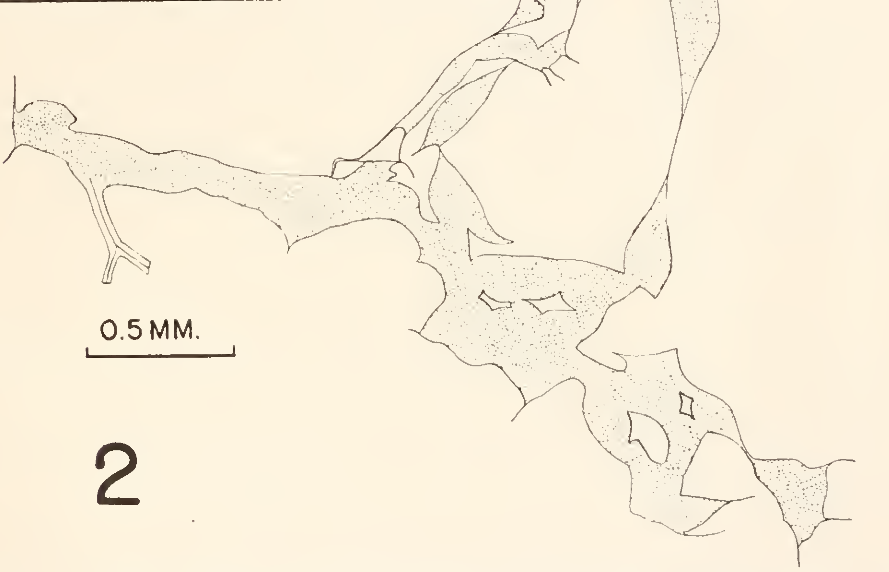


FIGURE 2. Living fragment of a prothoracic gland of a diapausing Cecropia pupa, showing branching and anastomosing structure

FIGURE 3. Highly magnified view of a fragment of a prothoracic gland of a diapausing Cecropia pupa. Fixed in Helly's solution and stained with Delafield's haematoxylin.

## DISCUSSION

It is not difficult to understand why prothoracic glands were overlooked for so many years. For, in the species that I have examined, the gland is a tissue rather than a compact organ. I regard complete or even partial excision of the prothoracic glands as technically incompatible with the survival of the animal. To secure individuals devoid of prothoracic glands, one must necessarily work with isolated abdomens.

Prothoracic glands are widely distributed among the Lepidoptera and, most probably, occur in all members of the Order (Lee, 1948). Preliminary, gross dissection has failed to reveal their presence in larvae of Diptera, Coleoptera, and Trichoptera, but they are conspicuous in the hymenopterous larva of the saw-fly, *Cimbex americana.*

Of special interest is the description by Pflugfelder (1938) of a bilateral glandular cell complex in the head region of the walking-stick, *Dixippus.* This complex was termed the "ventral head gland of inner secretion." It arises as an epithelial invagination in the region of the head and apparently extends into the prothorax where it is attached to the muscles and tracheae of this region. It attains maximal size just before the last moult, at which time the nuclei become lobulated. After the last moult the nuclear membrane is lost and the chromatin lies irregularly in the cytoplasm. The gland is degenerate in the adult. Pflugfelder suggested that the gland might play a role in nymphal development, but considered its extirpation to be impossible.

On the basis of this description, I propose that the ventral head glands of Pflugfelder are homologous with the prothoracic glands of Lepidoptera. If this is confirmed, then prothoracic glands must be present in certain hemimetabolous as well as holometabolous insects and the endocrinology of insect development must be re-examined from this point of view.

A careful survey of all Orders is required. Since the gland may conceivably vary in its structure and position, such a search should be directed towards a study of embryos with special reference to the presence and fate of invaginations of the lateral part of the second maxillary segment.

## SUMMARY

1. The data relative to the thoracic control of insect development are surveyed. Embryonic development seems to be controlled by a thoracic center in all insects. Postembryonic development seems to be controlled by a thoracic center in at least the Lepidoptera and the Megaloptera.

2. In the Lepidoptera the thoracic center for postembryonic development can be identified as the "prothoracic gland." A review is presented of the literature describing these organs.

3. The thoracic center for embryonic development coincides with the position occupied by the prothoracic glands in lepidopterous embryos. It is therefore possible that the prothoracic glands may function as the embryonic differentiation center in at least certain species.

4. The morphology and histology of the prothoracic glands are described in the Cecropia silkworm. Homologous organs are present in saw-fly larvae and most probably in the walking-stick, *Dixippus.*

5. Since prothoracic glands, or their homologues, may be widely distributed in embryonic and postembryonic stages of insects, these organs present special physiological interest and merit further study.

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# THE RELATION OF TEMPERATURE TO OXYGEN CONSUMPTION IN THE GOLDFISH[1]

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## INTRODUCTION

In general, studies on the relation of the metabolic rate to temperature have been limited to a consideration of the standard metabolism. Standard metabolism in animals has been taken as the approximate equivalent to basal metabolism in man. A distinction is made in deference to the fact that animals cannot approach the experiment in such a consciously basal state as can the human subject. However, from the ecological point of view, which recognizes that much of the measure of success which an animal may enjoy is due to its ability to be active, it is not enough to know the basal or standard metabolic state alone. It is of equal importance to measure the maximum metabolic rate, unless changes in the maximum rate follow no rule, or can be predicted from the standard rate. For fish at least there appear to be no data which establish either of these two provisos. For these reasons we undertook to explore the relation between a standard and a maximum level of metabolism in the goldfish and the ambient temperature. The two levels chosen were (1) a standard which was the minimum resting level in the diurnal cycle, a level as near basal as we could establish, and (2) a "maximum" which was the rate which appeared to be the highest the fish were able to maintain continuously until fatigue set in.

## MATERIAL AND METHODS

The goldfish used were a single lot of animals approaching one year old with an average weight of 3.8 gms. These fish were similar in age and source to those used for the exploration of various other physiological characteristics of the species (Fry, Brett and Clawson, 1942; Brett, 1946; Fry, Black and Black, 1947; and Fry and Hart, 1947). They were stored in running water and fed on a commercial fox food. Previous to any experiment the fish were acclimated to the particular temperature at which the experiment was to be carried out by maintaining them at that temperature for a length of time that would enable them to come to a stable state, as far as this is indicated by the stability of the upper lethal temperature (Brett, 1946). This period ranged from twenty days at 5° C. to less than four at 35° C. During this acclimation period the fish fed well. Both the standard and maximum levels of metabolism for any given temperature were measured on the same subjects, but the measurements at different temperatures were carried out on different samples drawn from the common stock. The metabolic rates were measured by determining the rate of oxygen consumption.

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[1] An abstract of this paper appeared under the same title in the *Anatonomical Record,* Vol. 96, No. 4 Supp., December, 1946.

All the rates of oxygen uptake were determined by means of the unmodified Winkler method. While nitrite contamination may introduce error into oxygen determinations (Allee and Oesting, 1934) it does not seem to have been a serious factor here. A number of checks with the Rideal-Stewart modification gave essentially the same readings as the unmodified Winkler's. Moreover, consistent results were obtained on check runs in which the initial levels of oxygen differed and hence similar levels were reached after different intervals of time.

Both the standard and the maximum levels of oxygen consumption were measured by determining the fall in oxygen content in closed containers (Fig. 1). The respiration chamber used to determine the standard level was a two-liter Erlenmeyer flask. The flask was fitted with a two-hole stopper through which projected two glass tubes (a and b), the inner ends of which were bent at right angles and spread well apart. A glass side arm (c) was blown into the Erlenmeyer at is widest diameter. This side arm was provided with a length of rubber tubing and a pinch cock. The fish were placed in water in the flask which was then placed on its side in a water bath with the glass tube uppermost. Tube (a) was connected to a second tube which led through the wall of the bath. Tube (b) was open to the bath. A supply of water of the desired temperature was led into the bath and aerated vigrously. Excess water passed out an overflow (d), thus maintaining a constant level of water in the bath. Water from the bath passed by tube (b) through the Erlenmeyer as long as (a) was open. The side arm (c) collected any bubbles which formed in the flask. Normally, tube (a) was left open, and the flow through the Erlenmeyer was so adjusted as to be sufficient to make the difference in oxygen content in the inflowing and outflowing water negligible.

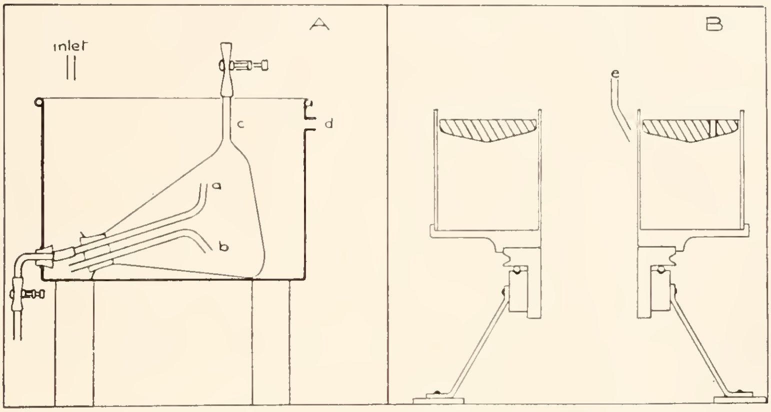

FIGURE 1. Arrangement of apparatus for measuring: A, standard metabolism; B, active metabolism. See text for detailed descriptions.

The chambers for measuring standard metabolism were set up in a basement room lighted by overhead lights in the daytime but dark at night, except for a shrouded fifty-watt bulb near the floor. The fish were in almost complete darkness at night since the metal bath shielded them from the light on the floor.

At temperatures of 20° C. and lower, twenty fish were placed in the Erlenmeyer; at higher temperatures the number was reduced to ten. The fish were placed in the chambers on the day previous to the experiment, and water samples for oxygen determination were taken as described below over at least one complete twenty-four hour cycle. All samples were taken as quietly as possible in order to minimize any disturbance of the subjects. However, at that time we were not conversant with the findings of Spoor (1946), and we undoubtedly did not eliminate such disturbance entirely. The minimum oxygen consumption generally occurred between 10 P.M. and dawn. The titration figures for each twenty-four hour cycle were plotted, and the lowest point during the night hours found by interpolation. The standard rate of oxygen consumption was calculated from the value thus found.

When a measurement of the oxygen consumption was to be made, any bubbles in tube (c) were carefully drawn out by suction. Water samples were taken from tube (a) and the bath. Tube (a) was then closed by the screw cock, thus isolating the water in the flask except for the connection through the relatively long and narrow tube (b). The time interval between the first and second sample was dependent on the metabolic rate. These intervals varied from four hours at 5° C. to thirty minutes at 35° C. The time between the initial and final samples was so chosen that the oxygen tension in the chamber did not fall low enough to affect the rate of oxygen uptake, a number of preliminary experiments being performed to confirm this fact. After the appropriate interval of time a second sample of approximately 100 cc. was drawn from tube (a) into a 50 cc. sample bottle, the first 50 cc. being used to flush, the second 50 cc. being retained as the sample. This partitioning of the sample was accomplished by taking the time during the first filling of the bottle and then allowing the water to flow for a further equal time before removing the sample bottle. It was presumed that this relatively rapid withdrawal of 100 cc. took place without any contamination from the corresponding 100 cc. of water which entered the Erlenmeyer from the bath.

The maximum level of metabolism was measured on fish swimming steadily against a current generated in a rotating chamber (Fig. 1B). This chamber was an annular vessel of rectangular cross section of which the outer wall was glass and the inner wall and bottom metal. The outside diameter was 12 inches, the width 3 inches, and the depth 6 inches. This apparatus is an elaboration of that originally used by Black, Fry and Scott (1939). A measured volume of water was placed in the chamber, the fish introduced, and the surface of the water protected from the atmosphere by means of a loosely fitting cover. Samples of water for the determination of the oxygen content were obtained by withdrawing 100 cc. by pipette. The pipette was then inserted to the bottom of a 50 cc. sample bottle and approximately 75 cc. of the sample was delivered into it, the excess being flushed out. The last 25 cc. in the pipette were discarded. Since the cover did not isolate the water completely from the atmosphere, some exchange of gas took place between the water and the air. The magnitude of this exchange at different temperatures and oxygen tensions was determined by filling the chamber with water which had been boiled and flushed with nitrogen, and then following the increase in oxygen with the cover in place. We are indebted to Miss J. M. Graham for making these determinations. A correction based on these results was applied to the apparent rates of oxygen uptake determined experimentally. The temperature of the water in the rotating chamber was controlled by playing a stream of water on its inner wall through tube (e).

## RESULTS

### (*a*) *Relation of temperature to standard metabolism*

The relation of the standard metabolism of the goldfish to temperature has been investigated by other workers on a number of occasions. On two occasions in particular (Ege and Krogh, 1914; Gardner, King and Powers, 1922) this relation has been worked out over a considerable range of temperature. The results of these workers are displayed with our own in Figure 2. The logarithms of the rates of oxygen uptake are used in this figure rather than the actual values so that a comparison can be made between the relative rates of change. The relative rates of change with increase in temperature are very similar in the three sets of data. Since different techniques were used in the three cases, such agreement can probably be taken as a satisfactory indication that the relative change is a stable characteristic of the species, although it must be remembered that this particular curve is characteristic of quite a number of other animals also (Krogh, 1941).

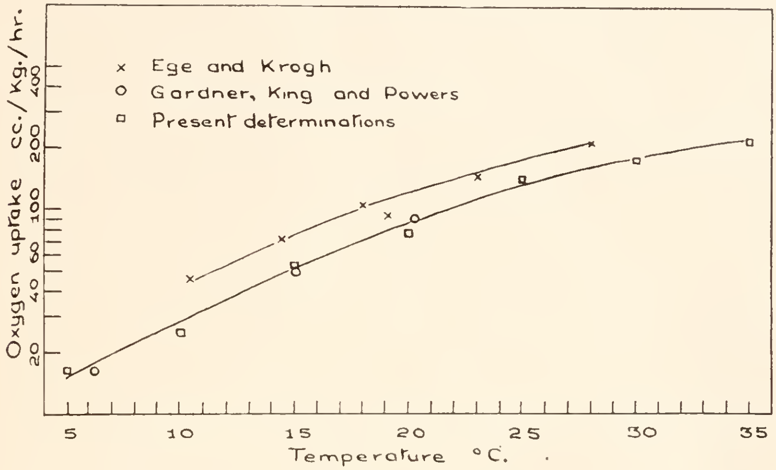

FIGURE 2. The relation of temperature to standard metabolism in the goldfish.

There does not appear to be any indication of any difference in this relative rate with difference in size such as wells (1935) believed was the case in *Fundulus parvipinnis*. However, his evidence is somewhat confused by the fact that the rates at different temperatures were measured at different times of day. Moreover, a good deal of the divergence in the rate-temperature curves he gives is an attribute of the magnitude of the absolute rates and disappears if a semi-logarithmic plot is used, as in Figure 2.

The rate of oxygen uptake per unit weight does not bear any consistent relation with size in the three sets of data in Figure 2. Such disagreement is probably due to the fact that the standard states used by the three groups of investigators were not the same.

### (*b*) *Relation of temperature and oxygen tension to the maximum rate of oxygen uptake*

So far as we are aware, no other workers have explored the effect of temperature on the maximum rate of oxygen uptake by the goldfish, nor have we examined this at all exhaustively, for we have confined our observations to what we believe to be the maximum level of oxygen uptake that can be maintained steadily.

The maximum rate was measured over a range of oxygen tensions down to those approaching asphyxial levels. In this way the effects of oxygen and temperature can be determined simultaneously and the scope of the experiment thus extended. The technique used was the familiar and simple one of allowing the organism to exhaust the oxygen in an isolated volume of water. In the case of goldfish it can probably be safely assumed that the small increase in carbon dioxide that results from the respiratory exchange in such experiments has no appreciable depressing effect on the rate of oxygen uptake. Under the conditions of our experiments the highest tension this respiratory carbon dioxide would reach in Toronto tap water is about 20 mm. at 35° C., a level which would appear to be far from any that would exert a limiting effect (Fry, Black and Black, 1947).

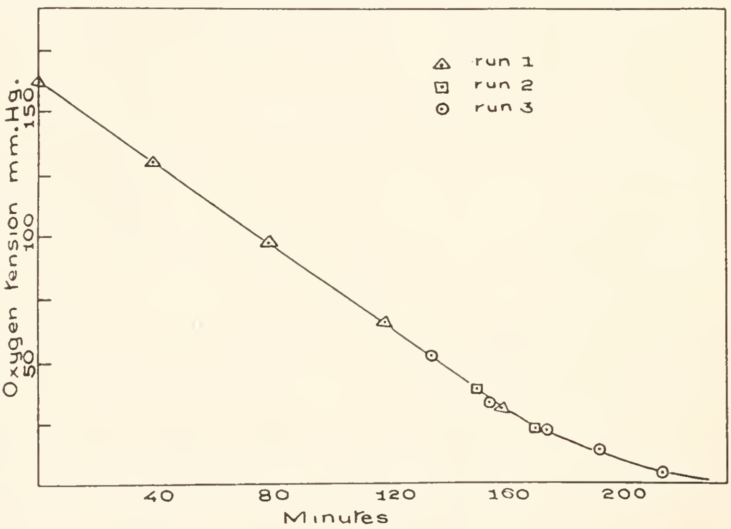

FIGURE 3. The time course of oxygen depletion in the rotating chamber, in a typical measurement of the metabolism of active goldfish (temperature 15° C., 20 fish, 4 litres water).

The course of the removal of oxygen from the water in the respiration chamber in a typical experiment is shown in Figure 3. Figure 3 presents the same picture as that found by Toryu (1927) for this species. Thus, our figures confirm his findings that the gold fish, at least when of the size of our subjects, has a respiratory system that is independent of the oxygen tension down to relatively low levels even under conditions of maximum continuous activity.

The maximum steady rates of oxygen uptake in relation to oxygen tension found at the different temperatures investigated are shown in Figure 4, where the rate of oxygen uptake is plotted gainst oxygen tension. These curves were derived by taking tangents from the appropriate graphs similar to Figure 3. Owing to the low level at which the oxygen uptake becomes dependent on oxygen tension, the data on which the descending portions of these curves are based are rather meager and no precise accuracy is claimed for them such as was attained by Maloeuf (1937) in his investigations. In particular, the curve determined at 20° C. is not consistent with the trend of the remaining data. However, although the proportional error may be considerable, the absolute error must be of no great order since the range of tensions occupied by this portion of the curve is in all cases less than 40 mm.

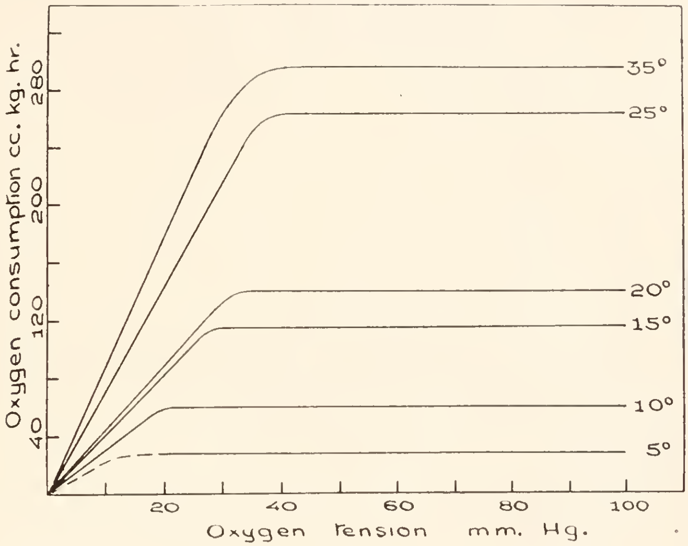

FIGURE 4. The relation of the maximum steady rate of oxygen consumption to oxygen tension at various temperatures.

While the physiological significance of the phenomenon commonly known as respiratory dependence has been often discussed at length since the beginning of the century, its ecological implications do not appear to ever have been clearly stated, except in connection with man in the case of the conquest of Mount Everest (Henderson, 1939).

The data in Figures 2 and 4 appear to us to offer two physiological indices that may be of great use to the ecologist. These have been designated by Fry (1947) as "the incipient limiting level" and "the level of no excess activity." The incipient limiting level is that level of oxygen tension below which the rate of oxygen uptake at the maximum steady state of activity begins to be reduced. This is the point of inflection of the curves in Figure 4. The incipient limiting levels of oxygen tension thus determined for the goldfish are given in Table I. The point of inflection in Toryu's data occurs at an oxygen tension of the order of these values, which appear to us to indicate that the respiratory rate he was measuring was well above the standard level under the conditions to which he was subjecting his animals. Such circumstances have been commonly remarked.

The level of no excess activity has been taken as that level of oxygen at which the animal can no longer satisfy more than its standard requirements for oxygen in spite of the utmost efforts of its ventilatory and circulatory apparatus. This level for the goldfish at a given temperature can be determined by finding on the curves in Figure 4 the tension corresponding to the oxygen consumption under standard conditions at the same temperature as determined from Figure 2. These values are presented in Table II.

TABLE I

*Tensions of oxygen at various temperatures below which the maximum steady rate of oxygen uptake is reduced in young goldfish acclimated to the temperatures at which the measurements are made*

| Temperature °C. | Incipient limiting level of oxygen mm. Hg |
|---|---|
| 5 | 15 |
| 10 | 21 |
| 15 | 29 |
| 20 | 34 |
| 25 | 39 |
| 35 | 40 |

TABLE II

*Estimated oxygen tensions at which young goldfish can take up no more oxygen than will satisfy the requirements of standard metabolism in subjects acclimated to the temperature at which the estimate is made*

The values for the standard metabolic rate in column 1 were taken from the smooth curve through our data in Figure 2.

| Temperature °C. | Standard met. rate cc. Kg. hr. | Level of no excess activity mm. Hg | Asphyxial level[1] mm. Hg | Residual level[2] mm. Hg |
|---|---|---|---|---|
| 1 | | | | 4 |
| 2.6 | | | nil | |
| 5 | 8 | 4 | | |
| 7 | | | | 4 |
| 10 | 24 | 8 | | |
| 11.4 | | | 8 | |
| 15 | 50 | 10 | | 4 |
| 20 | 85 | 18 | | 7 |
| 25 | 140 | 17 | | 8 |
| 27.2 | | | 12 | |
| 32 | | | | 8 |
| 35 | 225 | 25 | | |

[1] Gardner and King, 1922.
[2] Fry, Black and Black, 1947.

The levels of no excess activity given in Table II have, of course, been abstracted from a purely experimental situation, and the worth of such data to the ecologist must ultimately depend on proof that they have real significance as values limiting the activity of the organism in nature. The greatest uncertainty lies in the assumption that the standard level chosen was a fair approximation of the minimal respiratory requirements of the animal. We have not investigated the matter exhaustively, but a comparison is made in Table II with the values given by Gardner and King (1922) for the asphyxial level of oxygen for goldfish and what for convenience have been termed "residual levels" given by Fry, Black and Black (1947). These residual levels are the tensions of oxygen which remain in water in sealed bottles in which the fish have been allowed to exhaust the oxygen supply until they died. Thus, since some oxygen transport still goes on after the animals have been asphyxiated, and since the volume of water in which the fish were confined was small these residual values are somewhat below the asphyxial level. Taking the various circumstances into consideration, these three sets of data show fair agreement.

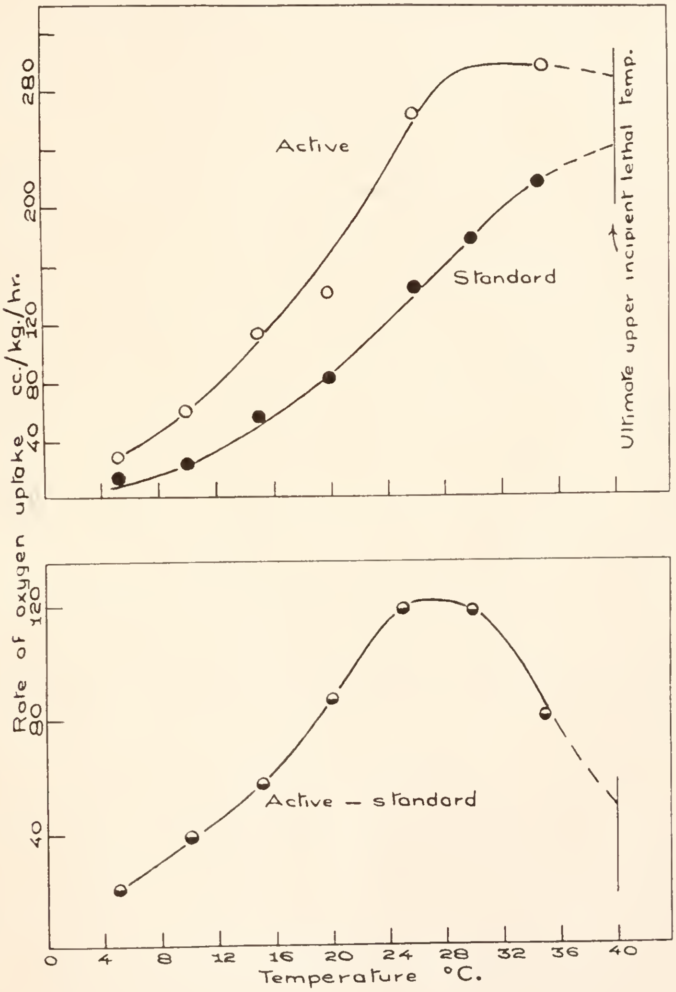

FIGURE 5. The relation between temperature and the active and standard levels of oxygen uptake.

### (*c*) *The difference between the maximum and standard levels of metabolism at various temperatures*

Assuming that the standard level of metabolism as measured here is a reasonable approximation of the maintenance metabolism, then the metabolism available for activity will be of the order of the difference between the maximum and standard levels. It will not be the whole difference, since with increasing activity the cost of ventilation and circulation and the other functions necessary to allow the external work to be carried on will, of course, increase, and some of the difference will be consumed for the increase of these auxiliary activities. The relation between temperature and the maximum rate of oxygen uptake at levels where the oxygen tension is not limiting, that is, at levels along the horizontal portions of the curves in Figure 4, is shown, together with the curve for standard metabolism in Figure 5.

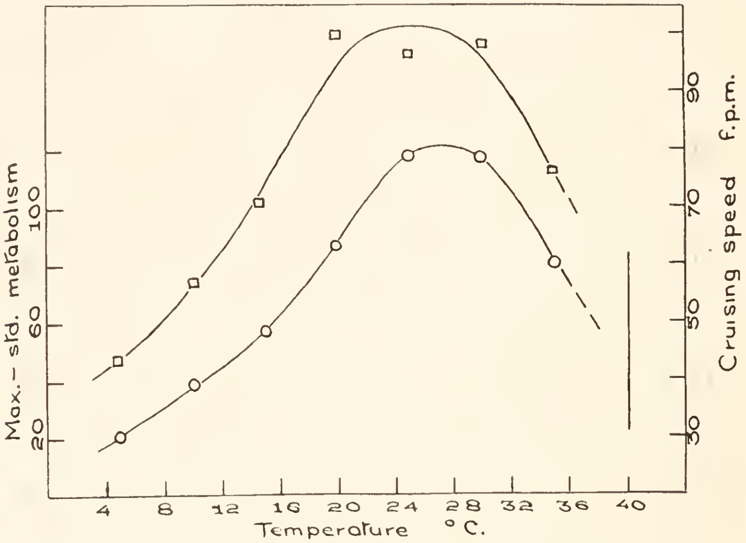

FIGURE 6. A comparison of the difference between the maximum and standard levels of oxygen uptake (lower curve) and the speed at which goldfish can swim steadily at various temperatures. The vertical line at 40° C. indicates the ultimate upper incipient lethal temperature of the goldfish.

In Figure 5 it will be seen that while the curve of standard metabolism probably increases with increasing temperature right up to the ultimate incipient lethal temperature (Fry, Brett and Clawson, 1942; Fry, Hart and Walker, 1946), the same is not true of the curve of maximum oxygen uptake. This latter curve reaches a height at about 30° C. which apparently represents the maximum capacity of the system involved, for it is not surpassed at higher temperatures. The consequence of the difference in course of the two curves representing the maximum and standard rates of oxygen uptake is that the difference between them reaches a maximum in the neighborhood of 28° C. and falls off rapidly above 30° C. The difference between the maximum and standard rates is given in the lower panel of Figure 5.

If the difference between the maximum and standard rates of metabolism does approximate the metabolism available for external work, then it would appear that there is an optimum temperature in the neighborhood of 28° C. at which goldfish can perform the most external work. This deduction from the data on metabolism is supported by observations on the relation of temperature to cruising speed (Fry and Hart, 1947). The curve illustrating the relation between temperature and the speed at which goldfish can swim steadily also appears to show a maximum between 20° and 30° C., although somewhat flatter than that for the difference between maximum and standard metabolism. The curves for cruising speed and the metabolic difference are compared in Figure 6. Thus, there seems to be the same correlation between oxygen consumption and activity over the whole temperature range that Spoor (1946) demonstrated at 23–25° C.

## DISCUSSION

The data presented on the maximum and standard rates of oxygen uptake point to an explanation of activity-temperature curves which drop at the upper end of the biokinetic range, which is alternative to the theory of thermal destruction usually invoked in such cases. The standard metabolism appears to increase with increasing temperature right up to the ultimate incipient lethal temperature. There is certainly no evidence of destruction or impairment of respiratory enzymes concerned with the standard metabolic rate up to a temperature of 35° C. On the other hand, the curve for maximum metabolism would appear to level off above 30° C. Such levelling off might be caused by a destruction of enzymes at higher temperatures to a degree which produces a balance between the effects of the increased temperature and the ability of the organism to respond, but some proof of this assumption is necessary if it is going to be offered as the explanation. Moreover, to explain the drop in cruising speed above 30° C. on the basis of thermal destruction of enzymes would then require that the explanation be applied to the maximum metabolic rate alone. However, a simpler explanation would be that the levelling of the maximum rate is because the capacity of some part of the organism concerned in oxygen transport is reached at about 30° C. However, whatever may be the explanation of the course of the curve of maximum metabolism, the correlation between activity and metabolism is not with either of the metabolic levels investigated here but with the difference between them. Or more properly, it is with some function of the difference between the two curves which ideally measure the metabolic levels and which our measurements approximate as is indicated by the correlation shown in Figure 6.

With respect to technique it appears that the simplest and most direct methods of obtaining ecologically significant values of oxygen uptake are to measure the maximum rate over a series of varying oxygen tensions down to the asphyxial level, and to measure the standard rate over a range of tensions at which oxygen would not be the limiting factor even for the maximum rate. In the case of the gold fish, for reasons stated above, carbon dioxide tension has not been considered, but for many other species it cannot be so ignored. In many respects however, there seems to be no major objection to combining the effects of carbon dioxide increase and oxygen decrease since they are combined in nature, particularly if the experiments are performed in water low in dissolved minerals so that a maximum increase in carbon dioxide tension will be attained for a given decrease in oxygen. Thus the limiting values for oxygen attained will approach the maximum that would be expected to be found in nature.

## SUMMARY

1. Two levels of oxygen uptake, (1) the lowest point in the resting metabolism in the daily cycle and (2) the maximum steady rate of oxygen uptake found when the fish were stimulated to activity in a rotating chamber, were measured at temperatures from 5° to 35° C.

2. The standard (resting) rate was measured over levels of oxygen high enough to avoid any dependence of the rate on oxygen tension. The maximum rate was measured over a series of oxygen tensions down to the asphyxial level.

3. The standard rate continued to increase with temperature up to 35° C., the highest temperature at which observations were made. The maximum rate was found by interpolation to reach its highest value at about 30° C. and to remain steady or decrease slightly at higher temperatures.

4. The maximum rate of oxygen uptake became dependent upon the oxygen tension between 15 and 40 mm. Hg, depending upon the temperature. These estimates were made in a closed system in which there was an accumulation of the carbon dioxide released in respiration.

5. Oxygen tensions at which the maximum oxygen uptake met only the needs of the standard metabolism were estimated to be between 4 and 25 mm. Hg over the temperature range investigated.

6. At the various temperatures the difference between the maximum and standard metabolic rates is correlated with the rate at which goldfish can swim steadily.

7. It is concluded that the drop in the sustained swimming rate of goldfish at temperatures from 30° to 38° C. is probably due to a decrease in the metabolism available for external work rather than to the thermal destruction of enzymes.

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# ADDENDUM

## SEMINAR, 5 AUGUST 1947, MARINE BIOLOGICAL LAB., WOODS HOLE, MASS.

*An experimental study of the 'second factor' in artificial parthenogenesis in the frog egg.* J. R. SHAVER.

Artificial parthenogenetic development of the virgin frog egg into cleavage stages and later embryos requires the introduction of cells or of cellular extracts, the 'second factor,' by pricking the egg with a fine glass needle, or by injection with pipettes at the time of activation of the egg (Bataillon, 1911 a, *C. R. Acad. Sci.,* T. 152, 920–922; Einsele, 1930, *Arch. f. Entwicklungs.,* Bd. 123, 279–300). Bataillon regarded the active principle of the inoculated cells as 'nuclear catalysts,' and Einsele thought the 'second factor' in watery extracts of frog blood and testis was a protein, possibly of enzymatic nature. The experiments here reported show that the injection into virgin eggs of granules and supernatant fluids obtained from homogenates of frog blood, testis, and of certain embryonic stages of the frog, by differential centrifugation, produces parthenogenetic development, the large basophilic granules acting as the 'second factor.' Tissues were extracted in M/200 phosphate buffer, slightly alkaline; granules were obtained by centrifugation of homogenates at 3000–6000 × g, and at 18,000 × g. The usual precautions against sperm contamination were observed. Small granules ('microsomes') obtained at 18,000 × g from blood, testis and frog blastulae gave smaller percentages of parthenogenetic embryos than the larger granules sedimented at 3000–6000 × g from testis and blastulae. Granules from stages earlier than blastulae were ineffective, as were the supernatant fluids from the high-speed centrifugations. Frog serum also produced cleavage when injected, but activity is markedly reduced upon high-speed centrifugation. The majority of parthenogenetic blastulae obtained show uncleaved areas, but the cleaved portions were seen to be nucleated in section. There is evidence that larger granules from tissue homogenates contain succinoxidase, cytochrome oxidase and other enzymes (Brachet and Jeener, *Enzymolog.,* 1944, **13**– 196; Chantrenne, *Biochem. et Biophysica Acta,* in press). It is suggested that the second factor may eventually be associated with one or more enzymes present in the granules, rather than with ribonucleic acid itself.

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# RETARDATION OF CELL DIVISION BY VITAMIN C IN PHYSIOLOGICAL CONCENTRATIONS

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## 1. On the Mechanism of the Action of Ascorbic Acid in the Body

Although numerous studies have been carried out on the gross and microscopic changes occurring in tissues in vitamin C deficiency, the mechanisms by which the effects are produced are still largely unresolved.

A survey of the pathological changes leads to the conclusion that the primary effect of ascorbic acid deficiency is an abnormality in the intercellular material, or perhaps at the cell surface, leading to changes in the bones and small blood vessels. In cases of this vitamin deficiency, formation of cartilage and bone is stopped, and the junction of epiphysial cartilage and bone is weakened. The widespread occurrence of hemorrhages points to the failure of the cells in the vessel walls to adhere normally and retain their functions as closed tubes, impervious to erythrocytes. This is due to failure in the formation and maintenance of intercellular materials and consequent weakness of the tissue (Wolbach and Bessey, 1942). The phenomena are of considerable interest to the cellular physiologist for they point clearly to some relation between cell structure and pathology, arising from a biochemical deficiency. Some of these questions of cellular action have been covered in a review by Reid (1943).

It appeared to be of some value to investigate the influence of l-ascorbic acid on cell division, because of the likelihood of some action on the cell surface, which might alter conditions there so as to produce an effect on cell division which can be measured quantitatively by known methods (Shapiro, 1941). Results to date indicate that ascorbic acid does not play a major role in tissue respiration since vitamin C depleted tissues show little reduction of oxygen consumption, and but a small increase when the vitamin is added (Stotz and Harrer, 1937). Stark, Gordon and Christensen state (in Elvehjem and Wilson, 1939), "it must be recognized that there has been assigned to ascorbic acid no respiratory function in animal physiology." This does not exclude the possibility that vitamin C might still play a minor role in cell oxidations, at the same time that it determines the form of the histological picture.

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Wolbach and Howe (1926) confirmed the fact that in scorbutus vitamin C is the only factor necessary for producing normal intercellular material. The deposition of homogeneous bone matrix was observed to make its appearance within a day of administering vitamin C to scorbutic guinea pigs. An inadequate ascorbic acid level in the body affects the collagen of fibrous tissues, bone dentine and cartilage matrices, and all non-epithelial cement substances, including that of the vascular endothelium (Wolbach, 1937). Another line of evidence hinting at the importance of vitamin C in cell division is that adduced by Phillips *et al.* (1940 and 1941). Ascorbic acid is concerned with the production of virile sperm in the bull, and is involved in the early phases of reproductive processes in the cow. Vitamin C administered to impotent bulls led to an elevation of the vitamin C level in both semen and blood plasma, and to a corresponding improvement in the breeding potency of the bull.

## 2. Agents Affecting the Rate of Cell Division

The rate of cell division may be altered by a variety of operations, e.g., by influencing the metabolic activity of the cell, the viscous and other intracellular forces, the cell surface, or by operating on a combination of these factors. Agents like cyanide, which profoundly depress cell respiration, will completely inhibit the cell division of the sea urchin egg. Calcium, on the other hand, will accelerate or retard cell division depending upon its concentration, and appears to act primarily on the cell surface (Shapiro, 1941). An absence of calcium leads to a disintegration of the embryo, occasioned by a falling apart of the cells (Herbst, 1900), and a general appearance of softness. With adequate calcium, the rapidly changing form of the embryo passes through its normal configurations, whereas with excess calcium, the rate of cell division is retarded. In this manner, if an effect on cellular metabolism can be eliminated, the influence upon the cell surface may be studied by observing the effect on the rate of cell division. The mechanism may be either a physical or physico-chemical one; in the case of the sea urchin egg, it was suggested that the calcium may combine quantitatively with protein at the cell surface according to a mass-action relationship, and thus alter the spatial relationship between these molecules in the surface and so change its mechanical properties (*cf.* also Zweifach, 1940).

## 3. Vitamin C Content of Normal and Malignant Tissue

Since cancerous tissue represents an abnormal growth with numerous cell divisions in a localized area, it is relevant to examine the results of studies of the effect of ascorbic acid administration and determinations of ascorbic acid content in such tissues.

Yovarsky *et al.* (1934), investigating the vitamin C content of various human organs, arrived at a range of from about 0.55 mgm. per gram for adrenal tissue, to about 0.40 mgm. for heart tissue, with wide individual variations. Musulin and collaborators (1936) using a dye titration method, found 0.61 mgm. ascorbic acid per gram of Philadelphia No. 1 rat sarcoma, with very little necrosis; 0.04 mgm./gm. in a human large liver carcinoma with severe necrosis; and 0.18 mgm./gm. in a human benign uterine tumor with slight necrosis.

Arloing *et al.* (1935) injected intravenously various dyes combined with vitamin C and ferrous and ferric chlorides. Occasional regressions of rabbit testicular tumors were observed, and in some human cancers of the tongue, tonsil, stomach and uterus, a retardation of growth and improvement in general condition. Severe edema was often produced near the tumor.

Boyland (1936) found that the indophenol-reducing potency of the Dael and Biltris transplantable guinea pig sarcoma is much reduced when the animal is maintained on a scorbutic diet. In this respect it behaves like normal tissues. When injected into scorbutic guinea pigs, ascorbic acid is selectively absorbed by the tumor tissue as well as by those tissues normally containing ascorbic acid. Ascorbic acid was found by Andervont and Shimkin (1939) to prevent the appearance of hemhorrage and ensuing regression of transplanted tumors treated with bacterial filtrate. They postulated that the bacterial filtrate lowered the ascorbic acid content of the tumor, and weakened its capillaries, with resultant hemorrhagic extravasation. Minor and Ramirez (1942) working with hospital patients, reported that the daily utilization of vitamin C averaged 67 mgm. for noncancerous patients, 68 mgm. for a patient with localized cancer, and 125 mgm. for patients with metastatic cancer. They suggested an accelerated usage of vitamin C by carcinomatous tissue. A. F. Watson (1936) had earlier found that the vitamin C reserves in the tissues of guinea pigs on a scorbutic diet are exhausted more rapidly if the animals are supporting rapidly growing tumors. Carruthers and Suntzeff (1942) observed that the ascorbic acid content calculated on the basis of weight of original tissue is significantly less in carcinomas than in the epidermis of normal mice, benzene-treated controls, or methylcholanthrene-treated mice. Although they found further that the ratio of ascorbic acid to nucleoprotein phosphorus was nearly the same for all groups, it does not abolish the significance of the differential ascorbic acid values found on the basis of wet weight. Robertson (1943), however, determined the ascorbic acid content of 22 tumors (of rat and mouse) and of comparable normal tissues. The ascorbic acid content of the tumors ranged from 15 to 70 mgm. per 100 gm. of fresh tissue. The ascorbic acid content of the tumors was not related to that of the tissues of origin. Robertson also concluded that no correlation between ascorbic acid concentration and rate of growth was apparent. Vogelaar and Erlichman (1937), studying the *in vitro* growth of Crocker mouse sarcoma 180, decided that vitamin C stimulates both the emigration of cells and the frequency of cell division, but furnished no quantitative measurements in substantiation.

In assessing the above results it should be borne in mind that apart from ascorbic acid, 2:6 dichloroindophenol will be non-specifically reduced by substances like stannous and ferrous salts, sulfites, sulfhydryl compounds, sulfides, thiosulfates, reductinic acid and "reductones," the latter formed by fermentation, or by splitting of sugars by heat at a suitable pH, and especially in the presence of protein (Farmer, 1944). These substances may have a structure similar to ascorbic acid with an aldol type of condensation between carbohydrate and protein derivatives.

## 4. Experimental Procedure

The following technic was adopted in order to measure the time required by fifty per cent of a population of cells to undergo first division in embryological development. Crystalline ascorbic acid (Eastman Kodak Co., Rochester, N. Y.)

was used and the solutions made up shortly before use.[1] The eggs of the sea urchin, *Arabacia punctulata*, were handled as in previous studies, e.g. (Shapiro, 1941). All eggs used in any given experiment were obtained from a single urchin in order to obtain the most homogeneous cell population available. 3 ml. egg suspensions were pipetted into a pair of 300 ml. Erlenmeyer flasks, forming a shallow layer on the bottom of each flask, to facilitate gaseous interchange. The flasks were immersed in a constant temperature bath at 26.1° C. ± 0.002° C., controlled by a thermionic relay. Thermostatic control is necessary since the rate of cell division is known to be markedly affected by temperature (Loeb and Wasteneys, 1911; Hoadley and Brill, 1937). After temperature equilibration (about ten minutes), the eggs in both control and experimental flask were fertilized at the same time by the addition to each flask of 6 drops of a previously prepared suspension of sperm in sea water, and both flasks were shaken while immersed, to distribute the sperm. After this the flasks were clamped in a stationary position. Oxygen was passed through sea water, and then over the shallow layer of eggs in each flask, to insure adequate oxygenation throughout the experiment. At regular intervals samples of the eggs were removed, and fixed for subsequent counts of per cent cleavage (cf. Fig. 1). At much later intervals, after the experiment, the eggs were examined to check for normal developments.

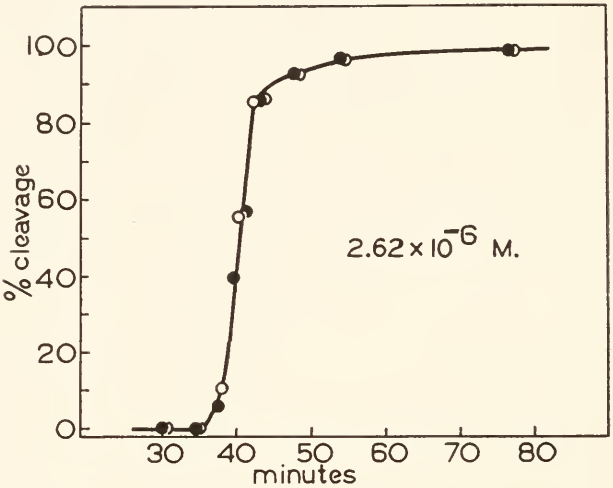

FIGURE 1. Cleavage curve showing the absence of effect at low concentrations of vitamin C, and the superposition of the two curves. Open circles, control eggs in sea water; closed circles, eggs in sea water containing $2.62 \times 10^{-6}$ molar vitamin C.

Where small effects on cleavage rate are encountered, it is essential to observe three precautions outlined above, in order to obtain reproducible results. Temperature must be controlled, adequate oxygenation insured, and the curve of cleavage as a function of time determined by an adequate number of samples, and plotted. Simple observation of eggs in syracuse watch glasses at the side of the aquarium may be satisfactory where gross retardation of many minutes is observed, but for optimal quantitative results the experience of the writer has led to the adoption of the above technic. Since a population of cells is being observed, with a statistical distribution of the time at which the cell divides, sigmoid curves of the types shown in figures one and two are obtained. The slope at the midpoint is variable, and depends upon the condition of the eggs, as well as the time in the breeding season when they are used.

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[1] Owing to the buffering capacity of sea water and the small amounts of vitamin C used, it is unlikely that the pH of the sea water was reduced from its normal value (approximately 8.1) to the region where pH is known to exert an influence on cell division (below pH 5.8, Smith and Clowes, 1924).

## 5. RESULTS

The data (Fig. 2 A–D) demonstrate clearly that vitamin C added to a suspension of eggs will delay cell division at all concentrations above a minimal one. The slowing down of cleavage is more effective the higher the concentration, but as evidenced in Figure 2 A–C, the cells are not prevented ultimately from undergoing the same total per cent cleavage as the controls. At the concentration shown in Figure 2 D ($1.57 \times 10^{-3}$ molar), not only is there a considerable delay in cleavage, but it appears that in approximately 22 per cent of the cells, cleavage is entirely inhibited. At very high concentrations of vitamin C the agent

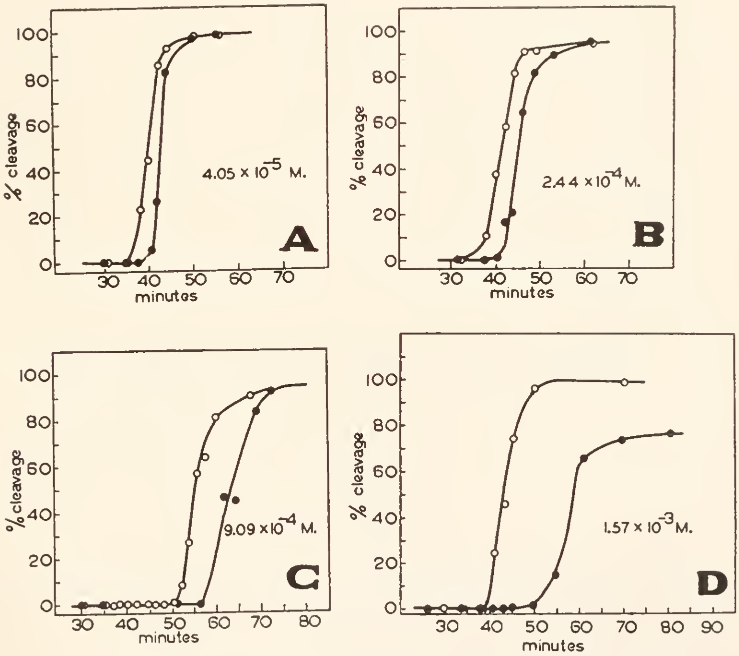

FIGURE 2. A, B, C and D show the increasing retardation of cell division with increasing concentration of vitamin C. The concentrations are given to the right of each pair of curves. Open circles, controls; closed circles, eggs in sea water with vitamin C.

becames toxic, and cell division can be inhibited in 100 per cent of the cells; moreover, the reaction leading to elevation of the fertilization membrane is likewise prevented. No concentration was found at which cleavage was accelerated.

In Figure 3, the amount of delay is plotted as a function of vitamin concentration. In the range studied (up to 277 micrograms per ml.), the relationship is a linear one, except at the very low concentrations, in the region of 0 to 5 micrograms per ml.

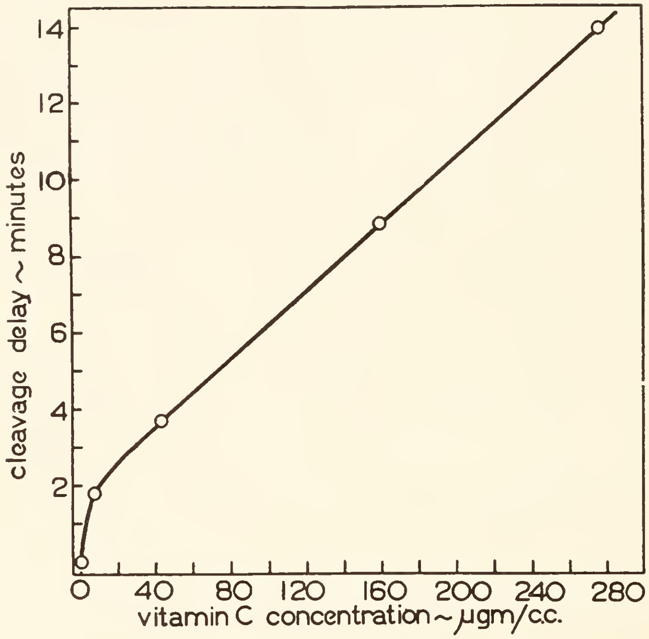

FIGURE 3. Concentration effect of vitamin C in retarding cell division.

## 6. SUMMARY

a. Eggs of the sea urchin, *Arbacia punctulata,* were exposed to concentrations of vitamin C in sea water, varying from $2.62 \times 10^{-6}$ molar to $1.57 \times 10^{-3}$ molar. The rate of cell division was determined by measuring the time required for 50 per cent of the cells to go through first cleavage.

b. Beginning with approximately $10^{-5}$ molar solutions, a definite retardation of cell division was observed. At high concentrations, complete inhibition of division occurred. At an intermediate concentration, the total percentage of cells dividing may be reduced from 100 per cent to some smaller figure.

c. Although cleavage was retarded in the presence of vitamin C, it appeared to be normal in other respects, with the exception of irregular cleavages at the high concentrations.

d. The slowing down of the rate of cell division appears to be roughly a linear function of the concentration, beyond a minimal concentration.

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# EXCYSTMENT IN THE CILIATE BURSARIA TRUNCATELLA

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## INTRODUCTION

The resting cyst of *Bursaria truncatella* was first described and figured by Cienkowsky (1855). Further observations were made by Brauer (1886), Bütschli (1887–89), Lund (1917), Penard (1922), Kahl (1932), and Poljansky (1934), so that its general structure is well-known. The cyst is spherical and has only two clearly recognizable membranes. Perhaps a third is present, since Poljansky, by treating the cysts with hypertonic salt solutions (method of Ilowaisky, 1926), was able to observe on the surface of the shrunken protoplast delicate folds which he interpreted as an innermost membrane or intimocyst. Whether these folds represent a valid membrane or a surface differentiation of the cytoplasm is not clear. The two readily observable membranes are a thin, inner endocyst and a thick, outer ectocyst whose relation to the inner membrane confers on the cyst its peculiar features of structure. The outer surface of the ectocyst is not smooth, but faceted. The center of each facet is depressed and is attached to the endocyst by a short, cylindrical bridge (Fig. 4). The bridges have been referred to by a variety of names, among them, Stäbchen (Brauer), Stränge and Faden (Bütschli), tubes (Penard), Balken (Kahl), and Füsschen (Poljansky). For brevity and accuracy Bütschli's description of the cyst (p. 1726) can scarcely be surpassed: "Cyste kuglig, mit doppelter Hülle; die innere an mehreren Punkten an der äusseren befestigt und letztere daher an diesen Stellen dellenartig eingezogen." Thus the ectocyst presents externally many roughly circular or polygonal depressions which are separated one from another by ridges. The ectocyst is apparently unattached to the endocyst in the areas between the bridges. It can be readily separated from the endocyst, according to Brauer, leaving the bridges attached to the latter. Thus the bridges appear to be derivatives of the endocyst.

One of the facets is somewhat larger than the others and bears what appears to be an unusually large bridge. Various names have been applied to this structure: Pfropf (Bütschli), hilum (Lund), collerette (Penard), and Cystendeckel (Poljansky). The use of these varied terms indicates that its true nature has not been clearly recognized. Its significance becomes evident only when the process of excystment is observed. Then it proves to be analogous to the micropyle of a Spongillid gemmule; it is an aperture of exit or emergence pore. Although the terms Pfropf and Cystendeckel suggest such a function, I have been unable to find in the literature a description of the actual excystment process. The present paper describes the process and includes some further observations on the properties and structure of the cysts.

## Material; Method of Inducing Encystment

In late August 1946, *Bursaria truncatella* appeared in considerable numbers in two large culture dishes in the laboratory. These contained the water, bottom sediment, and usual detritus of a recent collection from a temporary pool near Chapel Hill, North Carolina. With the addition of a little hay infusion from time to time *Bursaria* flourished in the dishes for a month, feeding largely on *Arcella, Colpidium* and *Paramecium bursaria* and dividing to all appearances exclusively by night. This peculiar habit of nocturnal division was noted by Schmähl (1926) and is unexplained. Evidently it is not correlated with the character of the food, since Schmähl's specimens fed largely on *Urocentrum, Stentor* and *Frontonia.* Individuals taken from the culture dishes during the day measured 400–550 μ by 225–325 μ and were therefore of average size for the species. Attempts to subculture them in smaller dishes and depression slides were unsuccessful.

Cysts were obtained by depriving active specimens of food in spring water. Usually groups of 10 or 20 individuals were removed to 0.75-cc. amounts of spring water in Columbia culture dishes. Again, groups of 50 were removed to 2-cc. amounts in Boveri dishes. After 24 hours the dishes always contained cysts exclusively, in number equal to or slightly less than the original number of specimens. Experience showed that the bursarias were easily injured by manipulation in small pipettes. Injured specimens disintegrated readily, and thus accounted for the disappearance of certain individuals. However, the losses from mechanical injury were low; for example, 13 dishes into which 260 bursarias were removed on August 30 contained 254 cysts on the following day. In general, one active specimen could be depended on to produce one cyst, lightly attached to the bottom of the dish. Since none remained unencysted, the encystment rate must be regarded as 100 per cent. Encystment usually occurred between the 6th and 12th hours of starvation. On many occasions a single active specimen was isolated in 0.75 cc. of spring water. A specimen so isolated always produced one cyst. Hence crowding, of primary importance in inducing encystment in *Colpoda* and *Didinium* (Barker and Taylor, 1931; Beers, 1947), plays no significant role in the encystment of *Bursaria.* Some 5000 cysts were obtained in August and September by the method outlined above. By means of a blunt needle they were detached from their original dishes and were stored in spring water in a smaller number of containers, some in stoppered vials, others in watch glasses kept in moist chambers. These cysts constituted the material on which the excystment experiments were based. Their history will be resumed shortly. At this point a further word on the factors responsible for encystment, followed by brief mention of some structural features of the cysts, seems in order.

Relatively little is known regarding the factors that induce encystment in *Bursaria.* Poljansky merely remarks that unfavorable conditions lead to encystment. Schmähl noted that specimens encysted after a variable number of days if kept at 4–7° C. in the presence of ample food, or if kept in continuous darkness, likewise with ample food. Hence, he cited low temperatures and darkness as encystment-inducing factors. However, neither of these factors was operative in the present study. Active specimens removed to spring water at 10:00 A.M. and kept at 22–24° C. in the natural light of the laboratory were always beginning to encyst at 5:00 P.M. Specimens removed to spring water at 5:00 P.M. encysted during the night. Changes in pH did not account for encystment, since the pH of the spring water (6.6) was practically the same as that of the culture fluid from which the bursarias were taken (6.4–6.8). Although the salt concentration of the spring water was undoubtedly well below that of the cultures, encystment could not be explained on the basis of a decrease in salt concentration for the following reason. When all the food organisms were removed centrifugally from a sample of fluid from one of the cultures, the bursarias encysted as readily in this fluid as in spring water. Hence, the evidence indicates that absence of food was the primary, and likely the sole factor responsible for encystment.

## Some Observations on the Structure of the Cysts

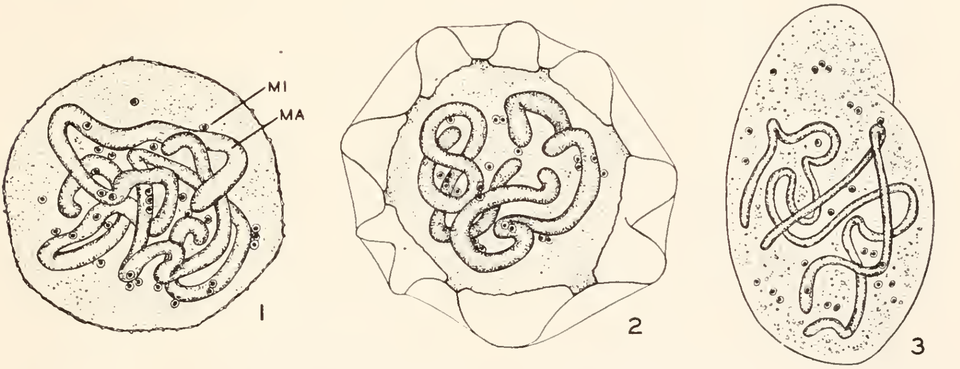
The diameter of the protoplast, as measured in the living condition, varied from 120 μ to 200 μ in the cysts at my disposal. Usually it was in the neighborhood of 155 μ when a sample of 20 or more cysts was measured. In Poljansky's cysts it averaged 167 μ. The thickness of the membranes was difficult to measure accurately because of their uneven external surface. It varied from 15 μ to 30 μ in different cysts; the mean was about 20 μ. Thus the membranes added about 40 μ to the diameter of the protoplast. The bridges usually measured 11–14 μ in diameter and 4–13 μ in length. As a rule they were short, but in some 4-month-old cysts they had a length of 15 μ and resembled short columns with broadened ends. Bridges of this type, but apparently even more elongated, are shown in Kahl's figure (p. 478). The number of bridges varied; some cysts had 40 or more. The diameter of the emergence pore varied from 20 μ to 30 μ. By reflected light the cysts appeared to be white. By transmitted light they looked black; actually they were opaque because of the immense number of granules in the cytoplasm. The granules were so plentiful that the macronucleus was quite obscured by them.

The emergence pore of the *Bursaria* cyst is closed by a thin, inelastic membrane (opercular membrane) which rests upon a low collar. Just beneath the emergence pore there is an area of hyaline, granule-free cytoplasm, though in my experience it is not always as conspicuous as the figures of Brauer and Lund indicate. Its significance is not understood, but since it is always prominent in the early stages of excystment (Fig. 5) it may be concerned with the elaboration of a substance which weakens the opercular membrane. It is evident that the emergence pore and underlying hyaline area give to the cyst an axis of polarity. Evidence deduced from the manner of emergence, to be presented later, indicates that the position of the emergence pore corresponds with the posterior end of the animal.

The nuclear structure and interrelations are well known for active bursarias, thanks to the work of Schuberg (1887), Schmähl, and Poljansky. The macronucleus has the form of a long, slender, variously contorted rod, whose total length often exceeds that of the animal. In some specimens it is in two parts. The micronuclei are small spheres, 4–5 μ in diameter and scattered indiscriminately in the cytoplasm. In stained preparations each appears as a central, deeply staining body surrounded by a clear area which is bounded externally by the nuclear membrane. Poljansky regards the clear area as a fixation artifact. The number of micronuclei is variable; Lund counted 9 to 15 or more; Schmähl, 20 or more; Poljansky, 15 to 34; my specimens had 16 to 28.

In order to examine the nuclear relations within the cysts, some of the active bursarias from the culture dishes were allowed to encyst on cover slips. These were immersed in Schaudinn's fluid (with acetic) at different stages of encystment and the specimens were stained by the Feulgen method. Individuals in the early stages of encystment remained attached and stained well. Individuals with fully developed cyst membranes usually became detached during the acid hydrolysis and had to be handled from this point in embryological watch glasses. The impervious nature of the mature cyst membranes introduced difficulties in staining, so that the final preparations of mature cysts were barely adequate to reveal the nuclei. Later, when it became possible to activate the cysts dependably, some of them were fixed and stained at various stages of emergence.

Whether a macronuclear reorganization occurs within the cysts was a point of special interest. If such a reorganization is present in ciliate cysts, it usually occurs in the early stages of encystment (Burt, Kidder and Claff, 1941; Beers, 1946). However, an examination of a considerable number of immature cysts (fixed 6–12 hours after isolation in spring water), as well as mature cysts (fixed 18, 24, or 48 hours after isolation), failed to reveal any evidence of a reorganization. Nevertheless, brief mention of the complex configuration of the macronuclear loops and of the number and spatial distribution of the micronuclei seems appropriate, since these features of the cyst have received little more than incidental mention in the literature.



Figures 1–3. *Bursaria truncatella.* Nuclear relations during encystment and excystment. From Schaudinn-Feulgen preparations. Camera lucida.

Figure 1. Young cyst. Macronucleus somewhat closely coiled; most of the micronuclei lying alongside macronucleus.

Figure 2. Immature cyst. All micronuclei now close to macronucleus. These relations are retained in the mature cyst.

Figure 3. Excysted specimen, immediately after emergence. Macronucleus in form of open loops; micronuclei scattered in cytoplasm. The macronucleus, whether in cysts or active specimens, may be in one part or in two.

In general the macronucleus retains throughout the encystment and excystment periods the type of structure already described for the trophic ciliate, although it assumes in the cyst the form of such close coils and loops that its parts may become actually intertwined (Figs. 1 and 2). Upon excystment the loops become more open, as formerly (Fig. 3). As to the micronuclei, they are scattered through the cytoplasm in pre-cystic (unattached or lightly attached) specimens, quite as in trophic individuals. As encystment proceeds, they assume a position close to the macronucleus, though distributed seemingly at random along its length (Figs. 1 and 2). This position is retained until excystment occurs, when they scatter (Fig. 3). There is no evidence of micronuclear division during encystment, and the number of micronuclei within the cysts agrees with the number found in active specimens.

## Attempts to Induce Excystment; Final Success

To return to a consideration of the cysts which were stored in spring water, many attempts were made between September 15, 1946, and June 1, 1947, to induce the emergence of samples of them. The procedure followed was similar to the one employed successfully in previous studies on the excystment of *Tillina* and *Didinium* (Beers, 1945, 1946a): 10 or 20 cysts were first washed in the medium to be tested and were then removed to 0.75 cc. of the medium in a Columbia culture dish. A detailed description of these experiments or a presentation of the results in tabular form would be little more than a wearisome chronicle of failure. The experiments will receive only brief consideration.

Samples of cysts stored at room temperature were tested when they attained the following ages: 1 week, 2 weeks; 1, 3, 5, 6, and 8 months. The following fluids were used: various concentrations of freshly prepared infusions of dried lettuce, some unbuffered, others buffered from pH 6.0 to 8.0 with phosphate buffer mixture, some at 22° C., others at 28° and 32° C.; similar concentrations of timothy-hay infusion, likewise varied with reference to pH and temperature; various concentrations of Difco yeast extract and peptone in aqueous solutions; the foregoing media pre-inoculated with wild bacteria; distilled water; spring water containing a dense population of *Paramecium caudatum*. Other samples were allowed to dry gradually on filter paper before being tested; still others were subjected to cold treatment (1 month at 15° C. followed by 3 months at 5° C.), in an effort to duplicate their natural winter surroundings. Of the dried and cold-treated cysts (about 500 of each were tested), none emerged. Of the cysts stored at room temperature (about 3000 were tested), the following 8 excysted. Three, age 1 month, emerged in unbuffered 0.1 per cent lettuce infusion at 22° C. and one of the same age emerged in similar infusion buffered at pH 8.0. One, age 3 months, excysted in 0.5 per cent lettuce infusion at 22° C. and 2, of similar age, in 0.2 per cent hay infusion at 28° C. Another, age 6 months, came out in 0.5 per cent lettuce infusion at 28° C. Excystment occurred between the 8th and 24th hours. It should be understood that a particular sample of cysts was tested in only one fluid and was then discarded, since an attempt was being made to discover a medium which had reliable excystment-inducing properties.

Even though the vast majority of the cysts failed to become active, I was not in the least disposed to regard them as dead. The dense granulation and the opacity of the cysts have been mentioned. The characteristic, uniformly opaque appearance was retained by practically all the cysts, with no indications of disintegrative changes. However, at least 6 of the 8 cysts that emerged, it was noted by chance, presented an exceptional appearance with reference to granulation and opacity. They had become less densely granular, light brown in color, and transparent to the extent that the macronucleus was faintly visible. They were assumed to be abnormal, and it was fortunate that they were tested for excystment and not discarded, for their emergence furnished the first tangible clue to a solution of the immediate problem. It indicated that cysts of *Bursaria* undergo with the passage of time a gradual, intrinsic physiological change which renders them capable of excystment. With this possibility in mind, the remaining cysts, about 750 which had been stored at room temperature and 250 which had received cold-treatment, were examined in the early part of June, but practically none showed a decrease in opacity.

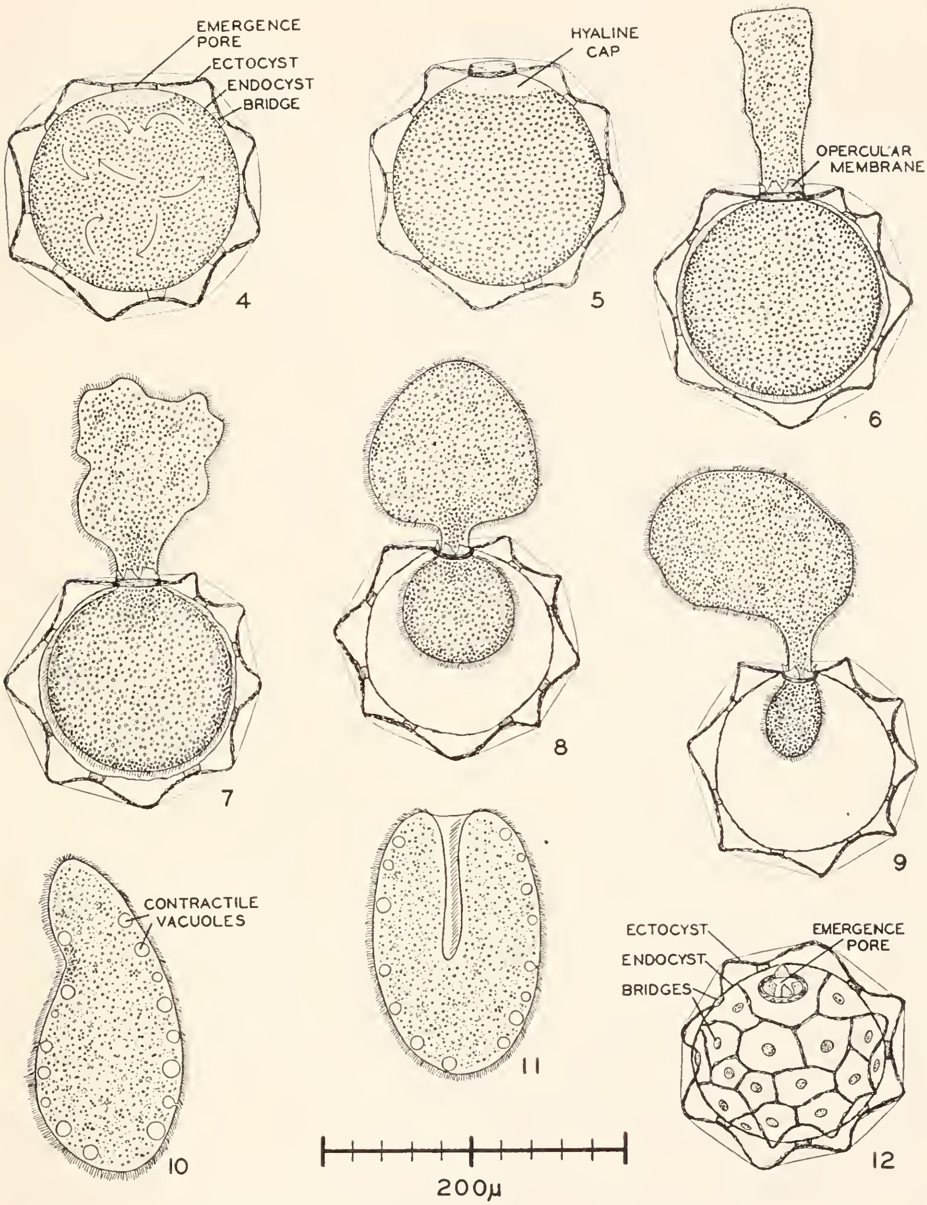
Shortly thereafter they were transported to the Mt. Desert Island Biological Laboratory, where they remained undisturbed until August 12. On this date, when they were about 11 months old, an examination showed that fully 80 per cent of both lots had become light brown and semitransparent in varying degrees. Samples of them, upon immersion in lettuce infusion (0.1 gm. dried lettuce boiled 5 minutes in 100 cc. distilled water, cooled and used at once), excysted regularly. Tests were made at 22° and 32° C. At either temperature practically 100 per cent of the cysts became active within 2–6 hours. Both lots responded equally well, so that the cold-treatment was without effect. Other samples tested in higher concentrations of lettuce infusion (up to 1 per cent) and in hay infusion (0.1–2.0 per cent) likewise excysted dependably.

With favorable cysts and suitable excysting media at hand, it became possible to observe the entire excystment process as often as desired. After an immersion of 2 hours in lettuce infusion in an open dish, the cysts were mounted in infusion under a supported cover slip and the sequence of events was recorded with the aid of a camera lucida.

## The Excystment Process

The excystment of *Bursaria* borders on the incredible. It is fully as amazing as the ingestion of *Paramecium* by *Didinium* or of *Closterium* by *Frontonia*. The details of the process follow, though it is understood that the time intervals cited may vary in different cysts.

Cyclosis begins after an hour in lettuce infusion and becomes more vigorous within the next hour. The cytoplasmic streams follow no regular path (Fig. 4). At the end of 2 hours the hyaline area under the emergence pore is conspicuously developed and resembles a transparent cap resting on the subjacent granular cytoplasm. Its base is always clearly delimited from the underlying granules (Fig. 4). The granules may oscillate near or stream by its base, but they do not enter it. It seems to be a region of gelated, granule-free cytoplasm. The opercular membrane now begins to bulge outward, and the collar of the emergence pore appears to be taller (Fig. 5). The cyst, as measured along its axis of polarity, has increased slightly in length at this stage. It is evident that considerable pressure is being exerted from within and that the emergence pore is the weakest area in the cyst wall. Suddenly the opercular membrane gives way and a column of cytoplasm protrudes eruptively (Fig. 6). In general the column is cylindrical, though its free end is sometimes broadened momentarily. The hyaline cap is lost sight of as the cytoplasm erupts, and the distal end of the column becomes highly ameboid. Indeed, the cytoplasmic column erupts so suddenly and changes shape so rapidly that this phase of the excystment process could be recorded faithfully only by cinephotomicrography, not by pencil and camera lucida. With the release of internal pressure the cyst membranes resume the shape of a sphere and a space appears between the endocyst and protoplast. The remains of the opercular membrane project outward as short jagged flaps from the collar of the emergence pore. Cytoplasm and granules now begin to stream through the emergence pore much as the plasmasol flows forward in an actively moving monopodal ameba. Thus the original column increases rapidly in volume, becoming bulbous and expanded in the meantime (Fig. 7). It changes shape constantly, rapidly extending and retracting numbers of lobose, pseudopod-like processes, as if in a state of great morphological instability, though the portion enclosed by the membranes remains spherical and relatively passive throughout the entire process of emergence. The presence of cilia can now be detected for the first time on the organism, and the cilia aid it in its further progress outward. By means of its cilia it retreats a short distance into its membranes and then delivers a vigorous thrust outward against the emergence pore. Each such outward thrust or lunge forces a little more of its surface area through the emergence pore, while its inner substance streams uninterruptedly through the pore. Thus two mechanisms are now operative in its emergence: ciliary action, which forces its cortical substance through the emergence pore by initiating vigorous outward thrusts; and endoplasmic streaming, which transports outward much of its inner substance, including the macronucleus. When the organism is approximately half excysted, it has roughly the shape of a dumbbell (Fig. 8). As the condition of final liberation nears, the free portion becomes unusually active. It changes shape constantly. At times it elongates, again it flattens on the slender, constricted, stalk-like portion and assumes the shape of the cap of a mushroom. It bends, first in one direction, then in another (Fig. 9), and twists through as much as 180 degrees on the stalk. Thus it becomes incredibly distorted, but as the last portion slips through the emergence pore these contortions end and it becomes pyriform in shape (Fig. 10). The first portion to emerge constitutes the rounded body, the last portion the neck of the pyriform ciliate. After a brief pause, the ciliate swims away, always with the pointed end, which emerged last, directed forward. Thus the posterior end lies immediately beneath the emergence pore and is the first part to emerge, whereas the anterior end emerges last. The contractile vacuoles show with great clarity now, and they are functional. Forty to 80 may be counted with ease, and there are probably many more. There is no recognizable indication of a ventral furrow or peristome at this stage, although some of these structures are no doubt represented in a specialized area of cytoplasm which occupies a sort of notch at the base of the neck. At this stage (Fig. 10) the ciliate could never be recognized as a member of the genus *Bursaria*. During the next hour, while the ciliate spirals counterclockwise through the medium, with brief periods of rest, its development is completed. Its pointed anterior end broadens, its body becomes flattened, and the ventral furrow and peristomial membranelles develop (Fig. 11). When these changes have been completed, the ciliate is ready to begin feeding. If food is not supplied, the excysted specimens re-encyst, forming smaller, light brown, semi-transparent cysts whose protoplasts measure only 95–120 $\mu$ in diameter. Following emergence the old cyst membranes are left intact in the medium (Fig. 12), and the number of bridges can be counted with relative ease.


EMERGENCE PORE
ECTOCYST
ENDOCYST
BRIDGE
4
HYALINE CAP
5
OPERCULAR MEMBRANE
6
7
8
9
CONTRACTILE VACUOLES
10
11
ECTOCYST
EMERGENCE PORE
ENDOCYST
BRIDGES
12
200μ

FIGURES 4–12. Excystment in *Bursaria truncatella.* The figures show successive stages in the emergence of the same individual; drawn from life with the aid of the camera lucida.

FIGURE 4. Cyst after 2 hours in excystment fluid. Arrows indicate direction of cyclosis.

FIGURE 5. Organism almost ready to break through emergence pore.

FIGURES 6–9. Escape by way of emergence pore.

FIGURE 10. The ciliate just after emergence—pyriform in shape and lacking ventral groove and membranelles.

FIGURE 11. Ventral groove and membranelles developing.

FIGURE 12. The empty cyst membranes.

The entire process of excystment and the subsequent differentiation require a minimum of 3 hours, counting from the time of immersion in the excystment medium. As has been said, 2 hours in the medium are usually necessary before the opercular membrane ruptures. Actual escape from the membranes requires only a few minutes. Thus the events shown in Figures 6–10 (rupture of the opercular membrane to final emergence) consume as a rule only 4 or 5 minutes. Subsequent differentiation requires 45 minutes to an hour. The foregoing time intervals are subject to much variation. Some cysts may remain 5 hours in the excystment medium before the opercular membrane tears. Some individuals require as much as 12 or 15 minutes to effect their escape. In general, it may be said that the time required for excystment at 22° C. varies from 2 to 6 hours.

## Further History of the Cysts; Re-encystment and Re-excystment

On September 1, 1947, there remained in the vials about 200 opaque cysts which presumably had not yet attained the physiological condition necessary for excystment. These were taken back to Chapel Hill and were set aside until October 18. When they were examined on this date, all were light-brown in color and semi-transparent. They were divided into 10 groups of approximately 20 cysts each. On October 20 and 22 five groups were tested for excystment in each of the following fluids: 0.2 per cent aqueous peptone solution; 0.1 per cent aqueous yeast-extract solution (Difco); 0.2 per cent lettuce infusion, freshly prepared; 0.3 per cent timothy-hay infusion, freshly prepared; Pyrex-distilled water. Some of the results were somewhat unexpected. Not only did all the specimens excyst in the plant infusions and in yeast extract, as expected, but all of those in peptone solution and distilled water also excysted. The time required for excystment varied as usual from 2 to 6 hours. Because of the relatively small number of cysts available for use in these experiments, it was impracticable to determine statistically which of the five fluids was most effective, i.e., which induced the highest percentage of excystment in the shortest time. Inspection of the results indicates that the peptone solution was least effective; none of the specimens in peptone solution was active at the end of 2 hours, whereas 20 per cent or more were active in the remaining fluids at the end of this time.

Many of the individuals which excysted in distilled water burst soon after emergence and all swam sluggishly. They were discarded. Most of the remaining specimens were transferred from their respective excystment fluids to a mixture consisting of 9 parts of spring water and 1 part of 0.1 per cent lettuce infusion, in which they encysted in the next 24 hours. This mixture was selected because its pH and tonicity are generally favorable for ciliates, and it does not support intense bacterial growth, which is often deleterious to ciliates. There resulted about 100 small, light brown, semi-transparent cysts. When these cysts were one week old, they were tested for excystment in the five fluids mentioned in the preceding paragraph. Practically all of them excysted after 2 to 6 hours in these respective fluids. Thus they did not require a long period of rest in order to become excystable.

## DISCUSSION

Reference to the work of Poljansky shows that there are two major categories of resting cysts in *Bursaria truncatella*. These are the so-called neutral cysts and the exconjugant cysts, the latter being formed by exconjugants when food is lacking. The two types are essentially alike as regards shape, cytoplasmic structure, and character of the membranes. They differ in the structure of the macronucleus. The exconjugant cyst contains 4 spherical or ovoid macronuclear anlagen; they are derived from 4 of the 8 nuclei that result from the first 3 divisions of the synkaryon. Neutral cysts are produced by vegetative individuals and therefore contain the usual elongated, bent macronucleus. Poljansky's exconjugant cysts were slightly smaller than his neutral cysts, probably because the exconjugants had had no opportunity to feed. Excystment was not studied by Poljansky.

I have had no opportunity to observe exconjugant cysts. All the cysts of the present study qualify as neutral cysts, a designation which is retained chiefly for descriptive purposes. The present results show that not all neutral cysts are alike in their capacity to excyst. Although the results are inadequate to permit conclusive generalizations, they show that there are two types of neutral cysts. Following the precedent of Johnson and Evans (1940) in their study of the cysts of *Woodruffia metabolica*, I shall designate them as stable and unstable. Probably this practice is warranted only for convenience of description; parallel comparisons of the cysts of the two genera are scarcely justified, for the cysts are very different structurally and somewhat different physiologically; their similarity resides chiefly in the fact that the unstable cysts become active more readily than the stable ones. Thus the *Bursaria* cysts which were obtained in late summer and early autumn of 1946 qualify as stable cysts. Stable cysts contain plentiful food reserves, to judge by their granulation. Presumably they are produced in autumn in nature when food becomes scarce, following a long period of vegetative reproduction in summer, though pure-line studies are needed to establish this thesis. They must pass through a long period of dormancy, in excess of 9 months according to the present observations, before many of them become excystable. During this period they undergo a physiological change which expresses itself visibly in reduced granulation and loss of opacity. Once a state of excystability is reached in consequence of these intrinsic changes, they excyst readily when a change in the chemical or physical nature of their environment occurs. Perhaps such cysts should be called winter cysts.

If the individuals which emerge from stable cysts are induced to re-encyst immediately by lack of food, they form unstable cysts. These contain fewer cytoplasmic granules and presumably meager food reserves. A long period of dormancy is not required to render them excystable; they can emerge within a week (perhaps sooner) if the environment is changed.

At this point in the discussion of unstable cysts, the results of some preliminary, though nevertheless significant, experiments on *Bursaria* cysts need to be recounted. In early May, 1946, there appeared in collections from the pool mentioned earlier a moderate number of specimens of *Bursaria truncatella*. Some hay infusion was added and the bursarias survived in the dishes for 3 weeks, though they never became numerous. Some of the fluid from the dishes was passed through Whatman No. 43 filter paper to remove the food organisms, and some 40 bursarias were transferred to this fluid. They encysted within 24 hours, producing cysts of average size. To judge by my sketches, the cysts were light in color and semi-transparent; at least the sketches show the macronucleus and indicate relatively sparse granulation. The existence of two types of neutral cysts was unrecognized at the time. When these cysts were 6 days old they were tested for excystment. Of 20 cysts treated with distilled water, 18 excysted; of 18 treated with 0.05 per cent lettuce infusion, 17 excysted. The precise time required for excystment was not recorded, but it was less than 8 hours. Thus the cysts, although not derived from immediately excysted specimens but from active, dividing specimens in mid-spring, qualified as unstable cysts. In all probability they were derived from recently excysted specimens which had passed through relatively few generations since emergence from stable cysts. Perhaps the unstable cysts should be called vernal cysts.

To summarize, the evidence indicates that *Bursaria* naturally produces stable cysts in autumn after a long period of vegetative reproduction, and unstable cysts in spring or at other times when relatively few generations have passed since emergence. Further investigations, including intensive culture work, are needed to clarify these points. Too little is known about exconjugant cysts to permit comment on their excystment.

To return to a consideration of the factors that induced excystment in this study, the emergence of *Bursaria* in fluids as dissimilar as peptone solution, lettuce infusion, and distilled water is not easily explained. Since no doubt exists concerning the hypotonicity of distilled water, excystment in this fluid may be readily explained in osmotic terms: water enters the organism, probably by way of the opercular membrane, and thus initiates its emergence. In all probability the remaining four fluids were also hypotonic. If it be assumed that excystment in these fluids was likewise initiated solely by osmotic phenomena, i.e., by hypotonicity, it is implied that the spring water in which the cysts were stored was not hypotonic. This implication seems ill-founded, in view of the low salt content of spring water. To explain the facts it seems necessary to assume that the *Bursaria* cyst reaches a state of equilibrium with the environment at the time of encystment. When this state of equilibrium is disturbed by the entrance of any of a number of kinds of ions or molecules into the cytoplasm, the organism responds by excystment. On the other hand, it is possible that these ions or molecules, instead of having a specific effect on the cytoplasm, merely alter the permeability of the plasma membrane or opercular membrane or both, and thus initiate excystment by allowing water to enter the cytoplasm. From this point of view excystment in any fluid, unless demonstrably hypertonic, could be interpreted in terms of osmotic phenomena.

Since ciliate cysts in general are never wholly separated from their immediate environment by impermeable membranes, it is more or less self-evident that they must be in some sort of physiological equilibrium with the environment; otherwise they would not remain in the encysted state. Furthermore, they have the capacity to reach this state of equilibrium in different environments; otherwise their encystment would be too restricted to have survival value. This is a manifestation of the adaptive property of protoplasm. For example, *Didinium* can form viable, normal cysts in 2 per cent hay infusion, in natural spring water, or in purely inorganic "artificial spring water" (Beers, 1947). The encystment of *Bursaria* either in spring water or in the rich, organic infusion of a wild mass culture has been mentioned. Probably all ciliates have the ability to encyst under many diverse conditions.

As to the conditions of excystment, were cysts so highly specialized as to be capable of emergence solely under a restricted and highly specialized set of conditions, the survival of the species would be threatened, in that these conditions might rarely be encountered. Hence, cysts are able to become active under a variety of conditions, some unfavorable for growth, multiplication and survival, others favorable. Paradoxical though it may seem, it is a fact that an effective excystment-inducing medium is not always favorable for continued life. Thus *Didinium* excysts readily in peptone media in which the bacterial activity is so intense that the didinia are killed upon emergence (Beers, 1946a). *Tillina magna, Bursaria* and other ciliates excyst in distilled water, which contains no food and is so hypotonic that some of the specimens always burst upon emergence. On the other hand, these ciliates also excyst under favorable conditions—for example, *Didinium* in infusions containing paramecia as well as bacteria which serve as food for the paramecia, and *Tillina* in plant infusions which support the growth of its bacterial food-organisms (Beers, 1945). By excysting under a variety of conditions, some individuals perish, but others survive. It is doubtful that the excystment of free-living ciliates can always be explained in terms of the action of a few specific, excystment-inducing substances.

## SUMMARY

A supply of cysts of *Bursaria truncatella* was obtained in autumn by depriving active specimens of food in spring water. The cysts were densely granular and opaque. In the following months many attempts were made to induce the excystment of samples of them, but with practically no success. Examination of the remaining cysts when they were 11 months old showed that fully 80 per cent of them had become relatively sparsely granular and semi-transparent. They could be activated dependably in plant infusions which had been ineffective earlier. Two months later the remaining 20 per cent of the cysts became excystable, not only in plant infusions, but also in peptone solution, yeast-extract solution, and distilled water. The results indicate that in autumn after the passage of many generations *Bursaria* produces cysts which require a long period of dormancy before they become excystable. These may be called stable cysts, but not all cysts are of this type.

Specimens which emerged from stable cysts, if not fed, re-encysted. These cysts, as well as others which were produced by specimens collected in spring, did not require a period of dormancy, but could be excysted within a week or less. These may be called unstable cysts.

The excystment process is the same in either type of cyst. It involves the rupture of the membrane which covers a special emergence pore, and the escape of the organism by way of this pore. Excystment is a remarkable feat, in that the spherical, encysted bursaria, measuring about 155 $\mu$ in diameter, makes its way outward through a circular opening measuring only 25 $\mu$ in diameter. Upon emer-

gence, the organism is so different in shape and structure from a typical specimen that it could never be recognized as a member of the genus *Bursaria*. Its development is completed within an hour.

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# THE CILIATES OF STRONGYLOCENTROTUS DRÖBACHIENSIS: INCIDENCE, DISTRIBUTION IN THE HOST, AND DIVISION

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Most species of sea urchins harbor in their alimentary tracts a characteristic fauna of ciliated protozoa. As Kirby (1941) points out, these ciliates appear to be obligatory inquilines; at least there is no satisfactory evidence that they are normally free-living forms which become established accidentally in sea urchins. Although their occurrence is well known, their general biology has been only meagerly explored. Little is known regarding their host-specificity, mode of transmission, and affinities; their taxonomy is confused, and their structure and division are inadequately described. In fact, it appears that representatives of only three genera have been subjected to critical morphological examination. These are *Entorhipidium* and *Lechriopyla*, which were studied by Lynch (1929, 1930) with special reference to ciliation, cytoplasmic inclusions and the neuromotor system, and *Entodiscus*, which was similarly studied by Powers (1933b). The conjugation of only one of them, *Cryptochilidium echini*, has been described (Daïn, 1930), and none have been cultured. Thus the ciliates stand in need of much additional investigation.

The present study is a contribution to the general biology of the ciliates of *Strongylocentrotus dröbachiensis*, the north-ranging "green urchin" common to both coasts of this continent and to Europe. The study makes no claim to comprehensiveness, but is restricted to the aspects enumerated in the title.

The studies of Powers (1933a) have shown that in the Bay of Fundy region at least seven species of ciliates occur in the digestive tract of this urchin. Some of them are of invariable occurrence; others are found erratically. The seven ciliates and their present taxonomic status are as follows: (1) *Entodiscus borealis* (Hentschel, 1924). This large, common ciliate was first described by Hentschel under the name *Cryptochilum boreale* from specimens taken from *Echinus esculentus* in the Shetland Islands. Madsen (1931) also found it in *S. dröbachiensis* at Frederikshavn, Denmark. He removed it from *Cryptochilum* and created the genus *Entodiscus* to receive it. (2) *Madsenia indomita* (Madsen, 1931). This slender, abundant ciliate was first described by Madsen as *Entodiscus indomitus*. Kahl (1934) removed it from this genus and established the genus *Madsenia* to receive it. (3) *Biggaria gracilis* (Powers, 1933). This ciliate was described by Powers under the name *Cryptochilidium gracile*. Kahl transferred it to the genus *Biggaria*, which he founded in 1934. This ciliate and the aforementioned two are trichostomatous holotrichs belonging to the Entorhipidiidae, according to Kahl (1934). (4) *Plagiopyla minuta* Powers, 1933. This uncommon trichostomatous holotrich is referable to the Plagiopylidae. (5) *Cyclidium stercoris* Powers, 1935. This small hymenostomatous holotrich, a member of the Pleuronematidae, was first described by Powers (1933a) as *Uronema sociale*. In 1935 he found this name to be invalid, being a homonym of *Uronema sociale* Penard, 1922. Since a further study of the ciliate convinced Powers of its close relationship to *Cyclidium*, he renamed it *Cyclidium stercoris*. (6, 7) *Euplotes* sp. and *Trichodina* sp. These two ciliates, a hypotrich and a peritrich, respectively, were not identified by Powers as to species. They were of uncommon occurrence.

The present study concerns these same ciliates—in particular, five of them—as found in 182 urchins from Frenchman's Bay, which to all practical purposes is a part of the Bay of Fundy. The urchins were collected three or four at a time almost daily throughout July and August 1947, and were examined immediately in an effort to discover the normal distribution of the ciliates. As a rule the examination of the first urchin of each small collection was well under way within 10 minutes after removal from its natural habitat.

The method employed by Lynch (1929) and Powers (1933a) in opening their urchins (cutting around the equator of the test and removing the intestine to a dish) was found to be unsuited to the present objectives, and the following method was adopted as least injurious to the alimentary tract. The urchin is rinsed in running tap water to remove external free-living ciliates and is laid oral surface uppermost on the table. With strong, sharply pointed scissors a circular cut is made around the test about 5 mm. to the outside of its oral opening. The central piece, consisting chiefly of lantern, peristomial membrane and adjacent, excised ring of the test, is lifted out and detached by severing the esophagus. With sharply pointed forceps or small scissors the mesenteries which hold the stomach (inferior or oral spiral of the alimentary tract) to the oral surface of the test are severed, and the opening in the test is enlarged by making a second circular cut about 5 mm. to the outside of the first. The perivisceral fluid is pipetted out. Thus the entire alimentary tract is left in its normal position in the test. With good fortune the stomach is uninjured, though sometimes its oral wall is slit in one or two places. The intestine (superior or aboral spiral of the alimentary tract) and the rectum (short, straight aboral portion which runs more or less meridionally to the anus) are never injured. With small scissors incisions large enough to admit an ordinary pipette are made at intervals in the alimentary tract and samples of the enteric fluid are removed. These may be examined at once or spread out on cover slips for fixation and subsequent staining.

The ciliates of *S. dröbachiensis* are found chiefly in the rather abundant enteric fluid, though if the food pellets are of a loose texture, owing to the ingestion of filamentous algae, they occur in limited numbers within the pellets. I have never found them in the siphon, in the perivisceral fluid or elsewhere in the urchin. André (1910) states that he found *Euplotes charon* on the exterior and in the perivisceral fluid of *Echinus esculentus*, and *Cryptochilidium echini* in abundance in both the intestine and perivisceral fluid of *Echinus miliaris* and *Paracentrotus lividus*. Hentschel is of the opinion that these urchins were moribund or that the ciliates were introduced into the perivisceral fluid in opening the urchins. To open an urchin with the assurance that the thin-walled and sinuous alimentary tract is still quite intact so that no ciliates can escape into the surrounding fluid, requires not only extreme care but also a very thorough examination of the condition of the exposed alimentary tract. Whether André's urchins received the requisite care and subsequent examination is not clear. Lacking the assurance that these precautions were observed, I am disposed to doubt that the ciliates occurred normally in some of the sites in which he found them.

## Incidence of Infection and Distribution in the Host

Of the 182 urchins, whose tests varied in diameter from 3 cm. to 6 cm., all were infected with *E. borealis* and *M. indomita;* 181 were infected with *B. gracilis;* 98 with *C. stercoris;* 28 with *P. minuta;* 24 with *Euplotes;* and four with *Trichodina.* These observations appear to be in general agreement with Powers' findings which were made 17 years earlier, though Powers (1933a) gives no actual figures on incidence of infection, except for *P. minuta* which occurred in 10 per cent of the hosts. The present incidence of infection with this ciliate amounts to 15.4 per cent. Powers states that *E. borealis, M. indomita* and *C. stercoris* occurred "in great abundance," and that infection with *B. gracilis* seemed "universal." Presumably the percentage of hosts infected with *Euplotes* and *Trichodina* was small.

The total number of ciliates present in a specimen of *S. dröbachiensis* from Frenchman's Bay is unbelievably great. Evidently Madsen's specimens from Denmark likewise harbored intense infections, for he speaks of the ciliates as occurring "in ungeheuren Mengen." To obtain a reasonably accurate idea of the general intensity of infection, a number of ciliate counts were made in the present study. The number of individuals of each species was counted in seven samples of fluid from different regions of the digestive tract of each of 12 urchins. These samples were taken from the first, middle and last thirds of the stomach; from corresponding regions of the intestine; and from the rectum (one sample). Based on the experience gained from these counts, estimates were made of the intensity of infection in seven samples from each of the remaining 170 urchins. Thus the findings on the intensity of infection in different regions of the alimentary tract are admittedly estimates for 170 of the 182 urchins, but it is believed that these estimates are reasonably faithful representations of the actual facts. The examination of these samples also served to disclose any differential distribution of the ciliates which might prevail along the course of the digestive tract. The degrees of infection were classified somewhat arbitrarily as "heavy," "moderate," or "light." The term "heavy," as applied to a particular species of ciliate, means an infection amounting to 500–1000 or more individuals per 0.1 cc. of enteric fluid; "moderate" indicates an infection of 50–500 per 0.1 cc.; and "light," fewer than 50 per 0.1 cc.

In the following summary of the results of these examinations, the various species will be considered separately, beginning with *E. borealis,* which was found in every urchin. The stomach was infected heavily in 40 urchins, moderately in 126, and lightly in 16; the intestine, heavily in seven urchins, moderately in 59, and lightly in 116; the rectum was lightly infected in all of them. Thus it is evident that *E. borealis* was most abundant in the stomach, less abundant in the intestine, and of relatively scant occurrence in the rectum. The principal or preferred site of infection of *E. borealis* is clearly the stomach.

Considering *M. indomita,* which was also found in every urchin, none of the 182 revealed heavy infections of the stomach, though 18 of them showed moderate infections and 24, light infections. As to the intestine, 131 urchins showed heavy infections; 39, moderate infections; and 12, light infections. The rectum was always lightly infected. Thus *M. indomita* is primarily an inhabitant of the intestine. It may occur in the stomach in moderate numbers, and it occurs in the rectum in much reduced numbers.

Turning to *B. gracilis,* a very different type of distribution was encountered. This ciliate was never found in the stomach. It occurred as light infections in the intestine of only 16 urchins, though in these it was restricted to the last pouch of the intestine. However, it occurred as infections of moderate intensity in the rectum of 178, and as light infections in three. (Only one was uninfected.) Thus *B. gracilis* is clearly an inhabitant of the rectum, from which it may extend forward (orally) into the terminal intestinal pouch.

*C. stercoris,* present in 98 urchins, was seen in the stomach of only one, and then as a light infection. Eighteen urchins harbored heavy intestinal infections; 61, moderate infections; and 19, light infections. The rectum was lightly infected in all 98. Hence, *C. stercoris* is primarily an inhabitant of the intestine.

*P. minuta* occurred as light infections in the intestine and rectum of 28 urchins. In many of these urchins it was distinctly more abundant than in those examined by Powers, since he never found more than 12 specimens per host. Many of the present samples contained 10 to 20 specimens, and a single sample represents only a small portion of the enteric fluid. Hence, many of the urchins contained hundreds of specimens of this ciliate.

*Euplotes* likewise occurred only as light infections of the intestine and rectum. The samples of fluid from the 24 urchins which were infected usually contained only five to ten specimens per sample.

*Trichodina* was found in only four urchins, as extremely light infections of the intestine and rectum. Only one or two specimens per sample were found in these hosts.

These results show conclusively that the various kinds of ciliates are not distributed indifferently along the digestive tract but exhibit to some extent definite preferences for different regions. Actually the three regions under consideration do not have precise anatomical limits, but rather pass insensibly one into another. To what extent the regions differ physiologically is not entirely clear. It seems to be generally assumed that the entire alimentary tract participates in the functions of digestion, absorption, and transport of dissolved substances to the perivisceral fluid. However, Weese (1926) found that an extract of the stomach of *S. dröbachiensis* had a pH of 6.3, whereas a similar extract of the intestine had a pH of 6.6, and it has long been known that in *Echinus esculentus* glandular cells are more abundant in the stomach than elsewhere (Chadwick, 1900). Thus minor physiological and histological differences occur along the digestive tract, and the physiological differences are probably greater than the limited evidence indicates. At least the differential distribution of the ciliates points to such differences, for some of them are adapted to life in one region, some in another. *B. gracilis* shows the highest degree of selectivity in its choice of a habitat, for it is almost exclusively an inhabitant of the rectum. Whether the nature of the rectal flora, on which it presumably feeds, or the chemical and physical properties of the rectal fluid account for its localization is not clear. Only *E. borealis* occurs regularly in the stomach. Evidently it has a high degree of tolerance for the digestive juices of this region. The remaining five ciliates show a distinct preference for the intestine. Since the ciliates of all sea urchins escape regularly with the feces (apparently never as cysts, but in their usual trophic form), any ciliate which occurs in the stomach or intestine will also be found in the rectum. Hence, all seven species inhabiting *S. dröbachiensis* are found in this region. Powers (1933a) showed that the ciliates of sea urchins have considerable tolerance for sea water, and it is assumed that they are ingested in the trophic form by new hosts. However, sea urchins regularly engage in cannibalism, small, weak or injured specimens being readily consumed, even to the spines and test. Cannibalistic practices afford an obvious means of transferring the ciliates from one urchin to another.

It is important to mention that the foregoing distribution prevails only in well-fed urchins and that all the 182 urchins appeared to be reasonably well-fed. The stomach of each of them contained scores or even hundreds of food pellets, which consisted of algae, small specimens of the clam *Mya arenaria*, and unrecognizable debris. When urchins were kept in laboratory aquaria without food for a week, the stomach was always found to be empty of food and practically devoid of ciliates, whereas the intestine always contained many food pellets and ciliates. (Such urchins, if kept together, must be uninjured and fairly uniform in size; otherwise cannibalism develops.) When other urchins were kept without food for two or three weeks, the intestine, as well as the stomach, was found to be empty, and the food pellets and ciliates were restricted to the rectum, the ciliates in much reduced numbers. Thus the food pellets and the ciliates simultaneously shift aborally when food is withheld.

## Periodicity of Division

Upon examining a dozen or more urchins in the early part of July, I was especially impressed by two seemingly contradictory aspects of the occurrence of *E. borealis, M. indomita* and *C. stercoris:* first, their great abundance, and secondly, the absence of dividing specimens in populations of such density. *Trichodina, Euplotes* and *P. minuta* are excluded from immediate consideration, for they were found in such limited numbers that the probability of observing divisional stages was somewhat remote. *B. gracilis* is likewise excluded for the present, since it was found in division in practically every urchin. The absence of dividing individuals in the remaining three species was genuinely puzzling, and the search for them was intensified. Throughout July and August more urchins were collected, as usual at low tide, and examined immediately, not only during the day but at night as well, so that finally practically all the hours of the day and night were represented in these examinations. The findings with respect to division will be presented for each of the three species in turn, beginning with *E. borealis*.

Of 88 urchins collected in July, only one, a specimen taken at 2:00 A.M., July 27, showed *E. borealis* in division, though rather sparingly. Six smear preparations of the stomach contents of this urchin were fixed on cover glasses in Schaudinn's fluid and stained in Mayer's acid hemalum. These revealed some 2400 specimens of *E. borealis*, only 3 of which were dividing. Thus 88 urchins which undoubtedly contained many hundreds of thousands of individuals furnished an utterly insignificant number of dividing specimens. Of 94 urchins collected in August, five contained *E. borealis* in division. In three of four urchins collected at 10:00 A.M., August 9, it was dividing in great abundance both in the stomach and first third of the intestine. So plentiful were the dividing forms that every sample of 0.1 cc., when examined under the dissecting binocular, was seen to contain 25–50 individuals in division, and smears made of material from these hosts showed all the stages in the division process. Again on August 10, two of four urchins contained dividing specimens, though not plentifully. These were the last specimens seen in division, though daily examinations were continued throughout August.

These results indicate that division in *E. borealis* is a cyclical phenomenon—that short periods of intense divisional activity alternate with long periods of non-divisional life. The factors which account for this apparent rhythmicity in the reproductive activities of the ciliate are at present unknown. Perhaps a more extensive study of the entire urchin-ciliate relationship—preferably a study embracing the entire year—would supply an explanation. The ability of *E. borealis* to maintain itself in such great numbers in the absence of frequent divisions indicates a low death-rate in the alimentary tract and a low percentage of losses at defecation. (Counts of ciliates expelled with the feces, to be mentioned shortly, support the latter conclusion.)

Powers (1933b) also had difficulty in finding dividing specimens of *E. borealis,* and indeed found only three in all the living specimens examined. On the other hand, Hentschel, in a study of *E. borealis* from *Echinus esculentus,* does not mention any difficulty in finding dividing specimens in heavily infected urchins, though he had only three such urchins. However, the relation of *E. borealis* to *Echinus esculentus* is evidently different from its relation to *S. dröbachiensis,* since Hentschel found only ten infected individuals among 52 urchins, and seven of these were lightly infected. Powers (1933a) remarks that *E. borealis* seems to be normally associated with *S. dröbachiensis,* "but is able to infest *E. esculentus* when the two species of sea urchins inhabit the same locality."

The task of finding *M. indomita* in division was even more arduous and unrewarding than the preceding, in spite of the fact that this ciliate, as in Madsen's specimens of *S. dröbachiensis,* was far more abundant than *E. borealis.* Only one dividing specimen was seen in 88 urchins in July, although some 450 samples containing *M. indomita* were examined and 52 stained cover-slip preparations of additional samples were studied. Many of the fresh samples contained well over 1000 specimens and many of the stained slides had 300 specimens per cover slip. A second dividing individual was seen August 9. Finally, three of seven urchins examined on August 28–29 showed *M. indomita* in division in considerable numbers, and slides made on these two days sufficed to show all the stages of division. Thus *M. indomita* is also able to maintain itself in immense numbers for long periods in the absence of division. Again, this circumstance indicates that it is not lost in great numbers with the feces and that it does not perish readily in the intestine. When division finally occurs, it appears to assume the character of a "mitotic flare," affecting great numbers of individuals simultaneously in any particular urchin. Neither Powers nor Madsen mentions the division of *M. indomita.*

As has been said, only 18 of the 182 urchins harbored infections of *C. stercoris* which qualified as "heavy." However, some of these infections were extremely heavy, each sample containing a veritable swarm of cyclidia, and there were 61 "moderate" infections. Hence, this ciliate was present, both in fresh samples and stained slides, in sufficient numbers to reveal divisional stages had they been present. Nevertheless, not a specimen could be found in division in July. Then, for a period of six days, beginning August 11, *C. stercoris* was seen in division in nearly every urchin examined—in 19 out of 20, to be exact. However, the number of dividing specimens per urchin was small and not all the stages of division could be found. This outbreak of division appeared to subside on August 16, and no more dividing specimens could be found. Thus division in *C. stercoris* appears to be cyclical, or at least sporadic.

Finally, a word concerning the division of *P. minuta* and *B. gracilis* must be appended. Infections with *P. minuta* were always light, and it was found in only 28 urchins. Few examples of division could be expected in such light infections, and none were found in 27 of the urchins. Then, on August 25, one urchin disclosed a dividing specimen. This urchin was subjected to a very thorough examination, and a total of 12 dividing individuals were found in 20 samples of rectal and intestinal contents. Hence, such evidence as is available indicates that the division of *P. minuta* also assumes a rhythmic character. *B. gracilis,* on the contrary, could be found in division readily in any urchin and therefore gave no evidence of major rhythms which affect the entire population of an urchin. However, *B. gracilis,* unlike all the other ciliates, is predominantly a rectal inhabitant. Counts of the ciliates lost at defecation, to be discussed immediately, showed that it is lost in greater numbers than any other ciliate. Therefore, it would seem that it must remain in a state of constant division in order to maintain itself within its host.

## Ciliate Losses Accompanying Defecation

It is generally implied that the ciliates of sea urchins are lost in considerable numbers with the feces, yet I have found no reliable numerical estimates of these losses. An attempt was therefore made to arrive at an estimate by counting the ciliates which accompanied the escape of the fecal material. The feces of *S. dröbachiensis* are passed in the form of fairly firm, more or less spherical pellets which measure 1.0–1.5 mm. in diameter. The pellets are passed singly and never in rapid succession; each emerges very slowly. Hence, the amount of rectal fluid which is lost at defecation seems to be reduced to the minimum. The pellets themselves contain no ciliates. Thus the ciliates which are actually lost must slip out between the wall of the anal canal and the surface of the escaping pellet. It is evident that the nature of the pellets and the mechanism of defecation do not facilitate the loss of ciliates.

The estimates of ciliate losses were arrived at by collecting and examining not only the pellets themselves but also the sea water in the immediate vicinity of the emerging pellets. A freshly collected urchin was placed in a dish of sea water in which it could move about and defecate normally. When a pellet first made its appearance among the anal plates, the end of a pipette was brought quite near it and 1 cc. of sea water was taken up and transferred to a watch glass for examination. A second cc., or a third or fourth, was taken up as the pellet continued its outward progress; then the pellet itself plus an additional cc., and a final cc. after emergence, were removed. Thus, it was hoped, all the escaping ciliates might be captured for counting. If an urchin failed to defecate within a reasonable time, it was given a piece of clam to feed on; ingestion usually stimulated defecation. When an urchin was kept under observation longer than 30 minutes, the sea water was changed, or better, the dish was set in an aquarium table and a gentle stream of sea water was directed into it. Urchins soon become sluggish in the absence of ample oxygen. Seven urchins, each of which was kept under observation for 1 hour, expelled a total of 186 pellets, and a total of 84 ciliates, which were distributed as follows: *E. borealis,* 16 specimens; *M. indomita,* 18; *C. stercoris,* 16; *B. gracilis,* 33; *P. minuta,* one. The results indicate, therefore, that approximately one ciliate is lost per two fecal pellets and that on the average an urchin loses 12 ciliates hourly or 288 daily. A number of additional counts, based on observational periods of 20–30 minutes each, supported these conclusions. A daily loss of 288 ciliates per urchin, or a loss of twice or thrice this figure should pellets be passed in appreciably greater numbers, is relatively small, for a single drop (about 0.05 cc.) of enteric fluid may contain as many as 1000 ciliates. Bearing in mind that a single division doubles the number of ciliates, it is evident that infrequent divisions would suffice to compensate for the losses which accompany defecation.

*B. gracilis* was lost in greater numbers than any of its confreres, although it is by no means as plentiful in the alimentary tract as *E. borealis* and *M. indomita.* Its relatively severe losses are evidently correlated with its habitat, since it is the only strictly rectal ciliate of the entire fauna, and indeed extends quite to the anal opening. In this disadvantageous position, it is lost in significant numbers, and as a consequence must remain in a state of constant division, it would seem, in order to maintain itself.

## METHOD OF DIVISION

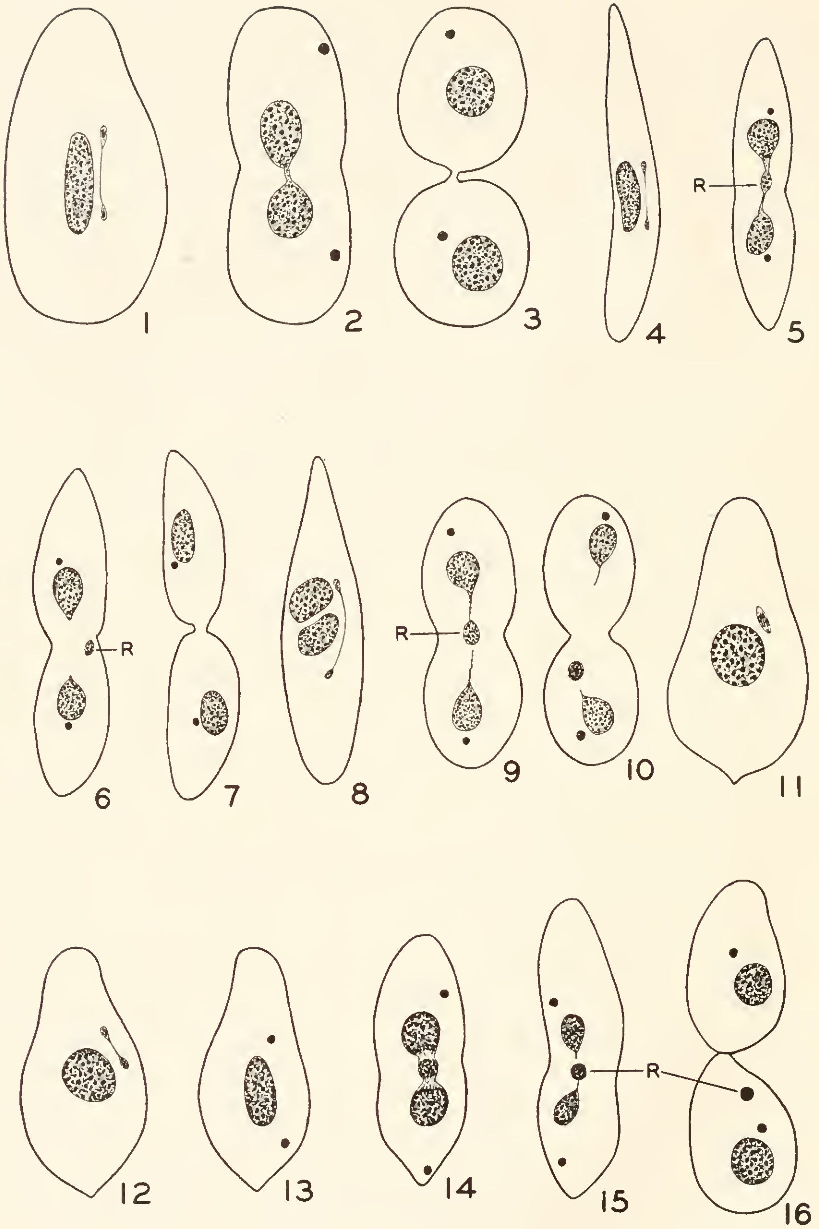
*Entodiscus borealis.* At division the single spherical or ovoid macronucleus elongates, and the micronucleus separates into halves with the production of a fairly long interconnecting strand (Fig. 1). The macronucleus then constricts cleanly into halves while the daughter micronuclei rest at opposite ends of the cell (Fig. 2). Each daughter macronucleus becomes spherical, and the posterior micronucleus begins to migrate to the usual position near the anterior surface of the macronucleus (Fig. 3). Thus division, as Hentschel remarks, "exhibits no remarkable features," but since he gives no figures of the process, three are included here.

Although the vast majority of the non-dividing specimens showed a single macronucleus, a limited number, as in those examined by Powers (1933b), contained a macronucleus which consisted of 2–7 parts, but always accompanied by a single resting micronucleus. In any particular individual of this type the parts were of approximately equal size, but the more parts per specimen, the smaller their size. Powers diagrams these multipartite macronuclei and gives measurements of their parts.

Issel (1903) observed that the macronucleus of various species of *Ancistruma* from marine pelecypods may be seemingly fragmented, often into 2–7 parts. Uyemura (1934) noted that the macronucleus of *Entorhipidium fukuii* from various urchins may be multipartite (in 2–8 pieces) and may be accompanied by two micronuclei, whereas there is typically only one. Uyemura concluded that nuclear conditions of this sort must be intimately related to cell division, though no dividing specimens could be found. Finally, Yagiu (1935), in a study of *Cryptochilidium ozakii* from Japanese urchins, likewise noted that the macronucleus may be in one part or in 2–7, but always accompanied by a single resting micronucleus.

Powers (1933b) interprets the multipartite condition in terms of a nuclear reorganization, and Yagiu regards the macronuclear elements as daughter macronuclei, since some of the large single macronuclei were lobed as if separating into smaller parts, but neither investigator seems genuinely satisfied with his own interpretation. However, the studies of Kidder (1933a,b) undoubtedly furnish the correct explanation of Issel's findings, and supply the probable explanation of the corresponding macronuclear conditions in *Entorhipidium fukuii, Cryptochilidium ozakii* and *E. borealis.* According to Kidder, the 2–7 macronuclei of *Ancistruma* are not actually fragments of a former macronucleus but are the macronuclear anlagen of a reorganizing exconjugant. In *A. isseli* from the mantle cavity of *Modiola modiolus,* Kidder (1933b) finds that the amphinucleus (synkaryon) of the exconjugant undergoes three divisions, from the products of which one micronucleus and seven macronuclear anlagen are derived. Three micronuclear divisions and three accompanying cell divisions follow. At these cell divisions the anlagen are segregated into the daughter cells as follows: at the first division, four into one daughter and three into the other; at the second division, two into each of three daughters and one into the fourth daughter which now has the typical nuclear complex and does not divide again; at the third division, one into each of six daughters. Thus specimens having one, two, three, four, or seven macronuclear parts may be found in a normal population of *A. isseli.* Although conjugation has not been reported in *E. borealis, C. ozakii* or *Entorhipidium fukuii,* it is logical to assume that it occurs, and reasonable to assume first, that it follows the pattern of *A. isseli,* and secondly, that the apparently multimacronucleate individuals are exconjugants which have not completed their reorganizational divisions. The schema outlined for *A. isseli* does not call for specimens with quinquepartite or sexpartite macronuclei, and these actually are almost non-existent in *E. borealis.* In some 5700 specimens Powers (1933b) found 162 which had multipartite macronuclei; they fell into these categories: bipartite, 103; tri-, 34; quadri-, 21; quinque-, O; sex-, 1; septem-, 3. In 4700 specimens from July and August collections I found 122 with multipartite macronuclei, distributed as follows: bi-, 79; tri-, 16; quadri-, 21; quinque-, O; sex-, one; septem-, five. No specimen in either collection had a macronucleus of more than seven parts. In accordance with expectations, if it be assumed that these specimens were exconjugants, bipartites predominated in both collections; three- and four-partites were plentiful and occurred in approximately equal numbers; five- and six-partites were either absent or so rare that they may be looked on as atypical; and seven-partites were the least common of all the expected types. If these are actually exconjugants, one would expect to find occasionally a specimen with a dividing micronucleus, yet neither Powers nor I could find even one. It is hoped that a further study of the ciliate will disclose the conjugants themselves and thereby show conclusively whether the present explanation is correct. In *Cryptochilidium echini* Daïn found that the synkaryon undergoes two divisions, thus producing one micronucleus and three macronuclear anlagen. Two segregation divisions follow, so that specimens having tri- or bipartite macronuclei occur normally.

*Madsenia indomita.* This slender ciliate contains a single, slightly elongated macronucleus and one micronucleus which usually lies just posterior to the macro-



Explanation of Figures

Figures 1–16. Division in four species of ciliates from the alimentary tract of *Strongylocentrotus dröbachiensis.* Schaudinn fixation; stain, Mayer's acid hemalum; camera lucida.

Figures 1–3. *Entodiscus borealis:* 1, macronucleus elongating, micronucleus in telophase; 2, division of macronucleus; 3, separation of daughters. × 225.

nucleus. At division the macronucleus elongates and the micronucleus divides typically (Fig. 4). The daughter micronuclei migrate to opposite poles of the macronucleus (Fig. 5), and the latter begins to constrict into halves. However, as the halves of the macronucleus draw apart, a small mass of macronuclear material remains as a residuum midway between the halves and within the attenuated nuclear membrane (Fig. 5). When the daughter macronuclei separate, the residual mass, which consists of little more than six to eight deeply staining granules in a lightly staining matrix, remains behind in the cytoplasm (Fig. 6). The life of the residual mass is brief, for it is resorbed before the daughter cells separate (Fig. 7). The elimination of a portion of the macronuclear substance at division is not of invariable occurrence; in some specimens the macronucleus divides cleanly with no suggestion of a residuum.

The accumulation of a mass of macronuclear material in the elongated nuclear membrane, its subsequent abandonment by the dividing macronucleus, and its final resorption in the cytoplasm have been described in four species of *Conchophthirius* (Kidder, 1933c, 1934) and in *Ancistruma isseli* (Kidder, 1933a) from pelecypods, and in *Cryptochilidium minor* from sea urchins (Yagiu, 1934). Any such material which in different ciliates is expelled by a variety of means from the macronucleus is usually looked on as effete and unwanted, and the process is interpreted as having a salutary effect on the macronucleus.

A very small percentage of the specimens of *M. indomita* contained a bi- or tripartite macronucleus, the parts being arranged usually in a series along the longitudinal axis of the ciliate. In two specimens which had a bipartite macronucleus, the micronucleus was dividing. One such specimen is shown in Figure 8. Perhaps these specimens were exconjugants undergoing their final reorganizational division.

*Cyclidium stercoris.* This is the smallest of the ciliates which occur abundantly in *S. dröbachiensis.* It has a spherical macronucleus which lies well toward the anterior end of the organism. The single micronucleus is usually found near the anterior surface of the macronucleus, but it may be at the side of, or just posterior to, the macronucleus. The scarcity of dividing specimens has been mentioned, and the early divisional stages were so elusive that none could be found in 32 stained cover-glass preparations which were made August 11–16. These preparations showed a number of specimens in the late stages of division and sufficed to demonstrate conclusively that the dividing macronucleus always discards a portion of its substance in the form of a residual mass within the attenuated macronuclear membrane. In Figure 9 the residual mass still occupies the mid-

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FIGURES 4–7. *Madsenia indomita:* 4, macronucleus elongating, micronucleus in telophase; 5, division of macronucleus with small mass of residual chromatin (R) at center of elongated nuclear membrane; 6, residual mass undergoing resorption; 7, separation of daughters. × 475.

FIGURE 8. *Madsenia indomita.* Micronuclear division in specimen with bipartite macronucleus. Probably the final reorganizational division of an exconjugant. × 475.

FIGURES 9–10. *Cyclidium stercoris:* 9, division of macronucleus with elimination of a conspicuous mass (R) of macronuclear chromatin; 10, residual mass free in cytoplasm. × 735.

FIGURES 11–16. *Biggaria gracilis:* 11, macronucleus spherical, micronucleus in metaphase; 12, micronucleus in telophase; 13, macronucleus elongating; 14, accumulation of residual macronuclear chromatin at center of dividing macronucleus; 15, residual mass (R) free in cytoplasm; 16, residual mass being resorbed, always in posterior organism. × 335.

point of the membrane, whereas in Figure 10 it is detached. The macronuclear behavior differs from that of *Cyclidium ozakii,* a ciliate of the echinoid *Anthocidaris crassispina,* for in *C. ozakii,* according to Yagiu (1933), the separation of the daughter halves is clean. However, even congeneric species are known to differ with respect to the extrusion of macronuclear material at division. Thus the dividing macronucleus of *Ancistruma mytili* never discards any of its substance, whereas the macronucleus of *A. isseli,* as has been said, always casts out a substantial residual mass (Kidder, 1933a).

*Biggaria gracilis.* The division of this striking ciliate, whose anterior half is distinctly flattened dorso-ventrally (using this term in a physiological sense) and whose posterior half is somewhat spherical and bulbous, offered no difficulty in view of the abundance of dividing forms. The macronucleus usually has the shape of an ellipsoid and lies transversely at the center of the organism. The single micronucleus is found just anterior to the macronucleus. With the approach of division the macronucleus becomes spherical and the micronucleus assumes a position antero-lateral to the macronucleus. In this location the micronucleus elongates and becomes spindle-shaped (Fig. 11). Then, with its long axis directed obliquely across the cell, the micronucleus completes its division (Fig. 12). I have examined at least 20 specimens in which the micronucleus was in various stages of division, and without exception it occupied the position just described. Thus the dividing micronucleus does not lie at the side of the macronucleus in the future cleavage plane of the cytoplasm, nor does the macronucleus begin to elongate until the micronucleus has completed its division. (In these aspects of nuclear behavior *B. gracilis* agrees with *A. isseli,* according to the figures of Kidder, 1933a.) The daughter micronuclei now move toward opposite ends of the cell, and the macronucleus begins to elongate (Fig. 13). Soon the macronucleus develops its divisional constriction, but in so doing a conspicuous mass of macronuclear material accumulates precisely at the center of the constriction (Fig. 14). This mass is of course the residual chromatin and associated substances which will be discarded when the macronuclear halves separate. A slight constriction appears in the cytoplasm at this stage. The daughter macronuclei now draw apart (Fig. 15), leaving the residual mass behind, while the cytoplasmic constriction continues to deepen. Finally, the residual mass passes into the posterior daughter cell where its resorption occurs, the posterior micronucleus migrates to its normal resting position just anterior to its macronucleus (Fig. 16), and the cells separate. The resorption of the residual mass is completed after the separation of the daughter cells. I have examined some 25 specimens in the final stages of division (Fig. 16), and without exception the residual mass was to be found in the posterior cell. In the four species of *Conchophthirius* and in *A. isseli* in which this type of macronuclear reorganization occurs the residual mass may be resorbed in either daughter cell, according to Kidder (1933a, c; 1934).

*Plagiopyla minuta.* Of the 12 dividing specimens which were seen, seven were successfully affixed to cover slips and stained. Unfortunately, Mayer's acid hemalum proved to be unsuited to the staining of *P. minuta,* since it stains the cytoplasm and food vacuoles of this ciliate intensely, thereby obscuring the nuclei. Owing to a scarcity of material, the Feulgen method could not be employed, and a consideration of division in *P. minuta* must be deferred. When applied to the remaining ciliates, acid hemalum gave excellent results, leaving the nuclei well stained against a faintly tinted background.

## SUMMARY

Of 182 specimens of *S. dröbachiensis,* all were infected with *Entodiscus borealis* and *Madsenia indomita*; 181 with *Biggaria gracilis*; 98 with *Cyclidium stercoris;* 28 with *Plagiopyla minuta;* 24 with *Euplotes* sp.; and four with *Trichodina* sp.

*E. borealis* occurs primarily in the stomach, though it extends into the intestine and rectum; *B. gracilis,* almost exclusively in the rectum; the five remaining species, chiefly in the intestine, less commonly in the rectum.

Although *E. borealis, M. indomita* and *C. stercoris* were present in immense numbers, they were rarely found in division. The evidence indicates that division in these ciliates, and probably in *P. minuta,* is a cyclical phenomenon; short periods of intense divisional activity appear to alternate with long periods of non-divisional life. *B. gracilis,* to the contrary, was dividing in nearly every urchin.

The ability of some of the ciliates to maintain themselves in enormous numbers in the absence of frequent divisions indicates that they do not perish readily in the alimentary tract and that they are not lost in great numbers at defecation. Counts of discharged fecal pellets and escaping ciliates indicate that an average of only one ciliate is lost per two fecal pellets. This rate of loss is low, in view of the great number of ciliates present per urchin. Thus infrequent divisions suffice to compensate for these moderate losses. *B. gracilis* is lost in greater numbers than any other species and must divide constantly in order to maintain itself.

At each division of *B. gracilis* and *C. stercoris* a mass of macronuclear material aggregates at the center of the elongated macronuclear membrane. This mass is discarded into the cytoplasm when the daughter macronuclei separate. A similar macronuclear reorganization usually accompanies the division of *M. indomita.*

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# THE BIOLOGICAL EFFECTS OF X-RAYS ON MATING TYPES AND CONJUGATION OF PARAMECIUM BURSARIA

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## INTRODUCTION

In the field of protozoology one of the most important and spectacular discoveries of recent times is the mating reaction leading to conjugation in *Paramecium* by Sonneborn (1937). If a single specimen of *Paramecium* is isolated and allowed to multiply so that eventually a great number of individuals are produced by vegetative fission alone, the group so derived from the one member is called a clone. Members of such a clone do not mate with each other because they are of one sex type but when one clone is mixed with another clone of the opposite sex type, the mating reaction will occur. In the mixture of the two sex types, the paramecia almost immediately agglutinate or clump together and subsequently conjugate. Upon mixing, a few are first seen to stick together then the clumps become larger and larger until 10–15 minutes later immense clumps of a hundred or so individuals are formed. These large masses then become smaller and smaller until later only small groups of individuals and joined pairs remain. Finally only individual pairs of conjugants (and single ones that were unable to find mates) are left.

Sonneborn discovered and described the mating reaction in *Paramecium aurelia* and Jennings (1938, 1939) described it in *Paramecium bursaria*. One is referred to their original papers for the detailed steps of the phenomenon. Additional information bearing upon the mating reaction and conjugation in *P. bursaria* is given by Wichterman (1944, 1946, 1948).

In an examination of the literature dealing with the biological effects of irradiation with x-rays upon *Paramecium* one is aware of the contradictory reports given by certain of the investigators. Part of the difficulty is due to the fact that earlier workers merely exposed the organisms to the action of x-rays and information upon unit dosage is absent. Without this information, it is impossible to make accurate comparisons of data. At the present time, with modern x-ray generators, it is possible to deliver rays of constant quality and intensity at a given number of roentgen units (*r*) per unit of time.

The main purpose of the present work was to find the effects of roentgen rays upon the mating reaction and conjugation of *Paramecium bursaria*. An abstract of this paper has been published (Wichterman, 1947).

## MATERIALS AND METHOD

In the experiments reported here, opposite mating types of *Paramecium bursaria* were used exclusively. *P. bursaria* is the so-called "green *Paramecium*;"

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green because of the fact that living within the cytoplasm of the protozoon are large numbers of extremely small unicellular green algae called zoöchlorellae which fill the body of *Paramecium.* There is thus shown a truly symbiotic existence in the relationship of algae to protozoon.

From eight different clones growing in the laboratory, two of opposite mating type were selected. These, called *C* and *D,* were received from the late Professor Jennings in 1940 and have been cultivated continuously. Organisms of opposite sex type when mixed show a feeble mating reaction in the early morning usually concomitant with the appearance of daylight. The reaction gradually increases in intensity and appears to be strongest at noon. The intensity of the reaction gradually decreases after 1:30 P.M., becoming feeble again at about 4 P.M. The mating reaction leading to conjugation does not occur in the clones under investigation between 4:30 P.M. and 5 A.M. The paramecia are readily cultivated in autoclaved lettuce infusion and grown in glass-covered flasks exposed to light coming from the north. Attempts were made during experimentation to reduce the entrance of bacteria to a minimum but relatively small numbers of bacteria of undetermined species were present which did not interfere with the mating reaction or conjugation. When paramecia are mixed at the proper time, the mating reaction and conjugation have never failed to occur in the seven years the cultures have been under observation.

Paramecia to be irradiated were selected from rich cultures which when tested gave the mating reaction. Concentrated paramecia were then placed in small celluloid boats measuring 4 × 2½ cm. and containing six cubic centimeters of their original clear culture fluid, then irradiated. Controls were used in every experiment. Paramecia for experimentation and controls were concentrated by simply removing them with a pipette from the upper surface of the cultures where, in daylight, they are found to congregate in great numbers.

The x-ray generator at the Marine Biological Laboratory is one of the most powerful in use for biological experimentation. It operates simultaneously two Coolidge tubes with a current through each of 20 to 30 milliamperes and an alternating voltage of 150 to 200 kilovolts peak. One tube is mounted rigidly near the floor and the other tube is supported on a counter-balanced arm which allows it to be moved vertically and in line directly over the fixed tube. The celluloid boats containing the paramecia to be irradiated were placed between the two tubes and were thus "cross-fired" by the two x-ray beams. Since the tubes can be brought very close together, the intensity of radiation upon the paramecia is extremely high, producing 6100 roentgens per minute. Furthermore, the distribution of radiation is more uniform than would be posible with a single tube. The two Coolidge tubes are water-cooled and since an electric fan was directed on the irradiated material, the slight rise in temperature was negligible during irradiation. Temperature was taken with a small thermometer whose bulb was placed directly in the boats of irradiated paramecia.

### *General effects of irradiation of Paramecium bursaria with x-rays*

It has been known for a long time that paramecia are able to survive exceedingly high dosages of roentgen rays. Irradiation of *P. bursaria* with 100,000 *r* results in greater activity of the organism. They swim faster than the controls, and unlike them do not settle on the bottom of the container.

With dosages of 200,000 *r* and higher, there is a noticeable retarding of motility until at those dosages approaching 900,000 *r* the animals show little movement although the cilia even at 1,000,000 *r* beat erratically. Even at dosages up to 900,000 *r* the paramecia swim in their characteristic manner in the form of a left spiral although in a wobbly fashion.

With dosages of 200,000 *r* and less, the effects of irradiation appear to be lost within a day and all or nearly all of the individuals survive and reproduce. This increase in numbers is seen when irradiated specimens are examined periodically each day for a week or more.

When irradiated with 300,000 *r* the paramecia at first move less actively than the controls but after a day swim actively. At this and lower dosages, the paramecia are capable of trichocyst extrusion when irritated.

An important effect of irradiation with 300,000 *r* and above is the destruction and loss of the zoöchlorellae. Two or three days after irradiation, the paramecia appear paler than before exposure to x-rays and eventually become colorless. Is the colorless condition due to the fact that the paramecia multiply while the zoöchlorellae multiply more slowly? This is hardly the case since roentgen rays inhibit division of the ciliate for a number of days after irradiation. Practically all of the contained zoöchlorellae appear to be destroyed as a result of irradiation but some may persist in a blanched or colorless state. The last green zoöchlorellae to disappear from irradiated specimens are the few that are lodged tightly against the cortex and close to the trichocyst layer.

Single isolations were made from pure-line mass cultures of colorless *P. bursaria* which were bright green with many zoöchlorellae before irradiation. These experimentally produced, colorless specimens have multiplied, resulting in pure-line mass cultures which have the same sex type as before irradiation and which readily mate and conjugate with specimens of the opposite sex type. They are being maintained in permanent culture, are still colorless and are being cultivated in flasks exposed to light in the same manner as the green races.

It is thus possible to obtain races of colorless *P. bursaria* which should prove to be useful in a number of experiments dealing with nutrition and symbiosis, serology, the mating reaction and conjugation. Such x-ray induced colorless forms are capable of living apparently indefinitely even though they change their type of nutrition from a purely holophytic or symbiotic one to a holozoic type in which they must now be dependent upon bacteria in the medium as food. Since it is evident that bacteria are killed in heavily irradiated cultures even though the protozoa are not (Brown, Luck, Sheets and Taylor, 1933), the author inoculated such irradiated cultures of colorless *P. bursaria* with bacteria known to support growth in other colorless races grown in the laboratory. In this manner the paramecia were prevented from starving. Such colorless clones are capable of demonstrating the mating reaction and conjugation with normal green ones of the opposite sex type.

From 400,000 *r* to 1,000,000 *r*, irradiation is seen to result in a decrease in numbers of paramecia. With irradiation of 400,000 *r*, 500,000 *r* and 600,000 *r*, it is possible to obtain survivors and begin new clones all of which are colorless and which later readily mate and conjugate.

Optically active crystals are not found in normal green *P. bursaria* but they are present in other species of *Paramecium*. In x-ray induced colorless *P. bursaria* crystals soon appear in fairly large numbers and the result is similar to that reported by the author (Wichterman, 1941) in which specimens of *P. bursaria* were placed in total darkness for long periods of time. Such specimens when placed in darkness lost most but not all of their zoöchlorellae, and optically active crystals appeared. However, when placed back again in light, the zoöchlorellae soon increased in number and the crystals completely disappeared. With *P. bursaria* irradiated from 300,000 *r* to 600,000 *r,* the green zoöchlorellae disappeared while the crystals became permanent structures even when kept in light.

When irradiated at these higher dosages, the survivors at first show great variation in size from the normal showing dwarfs measuring 64 $\mu$ in length and 24 $\mu$ in width to giant forms measuring 120 $\mu$ in length and 50 $\mu$ in width. Survivors of these higher dosages swim forward in a left spiral but in a slow, wobbly manner and appear to be fragile and easily broken. Cyclosis which is more rapid in *P. bursaria* than any other species is barely detectable in the survivors when examined immediately after being irradiated with 600,000 *r*. At this dosage small, clear, structureless vesicles form upon the pellicle which grow to a size of 8 $\mu$, then leave the body. A similar phenomenon was noted by Crowther (1926) in irradiated *Colpidium colpoda,* a ciliate closely related to *Paramecium.*

Irradiation with x-rays markedly increases the viscosity of the protoplasm; higher dosages lead to irreversible coagulation. Survivors that had been irradiated with 600,000 *r* are colorless individuals which later show the normally rapid cyclosis so characteristic of this species. Contractile vacuoles are seen with great clarity and the ciliates possess numerous crystals such as are found in other species of *Paramecium.* At the present time, colorless ones which were green and had been irradiated with 300,000 *r* and 600,000 *r* are flourishing in permanent culture in the laboratory.

One can obtain survivors from clones irradiated with 600,000 *r* and produce rich cultures but above this dosage there are no survivors. When irradiated at dosages of 700,000 *r,* 800,000 *r,* 900,000 *r,* and 1,000,000 *r* the mortality is seen to be exceedingly high soon after irradiation. None survive after the paramecia are transferred to fresh, unirradiated lettuce infusion even when inoculated with bacteria. With 700,000 *r,* paramecia swim very slowly and die in about five hours. With dosages of 800,000 *r* and 900,000 *r* the effect is similar with cytolysis occurring in many. Most specimens die within an hour after irradiation. When irradiated with 1,000,000 *r* and then examined immediately most, but not all, of the animals are seen to be dead. Dead or dying specimens appear swollen or spindle-shaped. The last sign of life that is observed is the movement of cilia which are seen to beat in an erratic manner.

### *Effect upon mating reaction and conjugation when both sex types are irradiated*

Individuals from highly reactive cultures of opposite sex type were irradiated at one time at a given dosage. The dosages ranged from 100,000 *r* to 1,000;000 *r* in steps of 100,000 *r*. The mating types were mixed immediately after irradiation then observed under a low power binocular microscope. The results at given dosages follow and are shown in Table I.

TABLE I

*Effect of roentgen rays upon locomotion, the mating reaction and conjugation of Paramecium bursaria*

| Dosage in roentgens (*r*) of two irradiated mating types of opposite sex (*C* and *D*) | Effect on locomotion | Effect on mating reaction and conjugation | | |
|---|---|---|---|---|
| | | Result when two mating types were mixed directly after irradiation | Day following irradiation when mating reaction first occurred | Day following irradiation when conjugation preceded by the mating reaction was seen to occur |
| 100,000 *r* | More active than normal | Mild mating reaction with small clumps of only 10 or less paramecia | 1st (immediately followed by conjugation) | 1st |
| 200,000 *r* | Slower than normal | Mild mating reaction with small clumps of only 10 or less paramecia | 1st (immediately followed by conjugation) | 1st |
| 300,000 *r* | Slower than normal | Mild mating reaction with small clumps of only 10 or less paramecia | 1st (no conjugation) | 2nd |
| 400,000 *r* | Slower than normal | As above but 45 minutes after mixing | 3rd, 4th and 5th (no data beyond 5th day) | —— |
| 500,000 *r* | Slower than normal | No mating reaction | 3rd day, then daily | Few pairs on 7th day and more on following days but most on 12–14th |
| 600,000 *r* | Slower than normal | No mating reaction | 3rd day, then daily; clumps increasing in size | Few pairs on 7th day and more on following days but most on 12th–14th |
| 700,000 *r* | Very slow moving | No mating reaction | Never | Never |
| 800,000 *r* | Very slow moving | No mating reaction | Never | Never |
| 900,000 *r* | Very slow moving | No mating reaction | Never | Never |
| 1,000,000 *r* | No locomotion but ciliary activity | No mating reaction | Never | Never |

### *a. Effect of 100,000 r*

Upon mixing immediately after irradiation small groups of two to five paramecia were formed which after approximately five hours dispersed into singles again. On the other hand, the controls when mixed at the same time showed huge clumps of a hundred or less individuals which ended in pairs undergoing conjugation. It was not until approximately six hours after irradiation that the paramecia settled characteristically on the bottom of the dish in their normal swimming manner. Apparently the effect of irradiation at this dosage is lost in this time, and all individuals survive. However on the following day there is a normal mating reaction with the formation of large clumps which now culminates in conjugation. It is clear that irradiation at this dosage prevents conjugation on the day of irradiation but does not stop a weak mating reaction from taking place.

### *b. Effect of 200,000 r*

The effect at this dosage is very similar to that mentioned above except that the paramecia before mixing are seen to move less actively than the controls while those irradiated at 100,000 *r* were more active than the controls. When opposite sex types were mixed, small groups of two to five paramecia formed which separated into singles again four hours after irradiation. On the day following irradiation the normal mating reaction occurred which was followed by conjugation.

### *c. Effect of 300,000 r*

When mixed immediately after irradiation, small clusters of paramecia are formed which separate into singles again. The mating reaction again occurs on the day following irradiation but the individuals leave the clumps as before. However on the second day following irradiation, the mating reaction occurs with the formation of larger clumps which now results in conjugation.

In dosages up to 300,000 *r* no appreciable lethal effects were noted. At this dosage and above, animals appeared blanched or pale green several days after irradiation. Later they become colorless. Clones started from isolated colorless individuals regularly demonstrate the typical mating reaction followed by conjugation when mated with normal green *P. bursaria* of the opposite sex type.

### *d. Effect of 400,000 r*

Upon mixing after this dosage there is no immediate clumping but after about 45 minutes, groups form of eight or more individuals. A few small clumps persist for about 10 hours after irradiation which subsequently break down into singles again. This mild clumping reaction occurred daily for the next five days following irradiation but did not result in conjugation during that time. Information is not available beyond this time. At this dosage there is a noticeable decrease in the population of paramecia in the cultures indicating a marked lethal effect. The survivors show little activity while the controls are very vigorous.

### *e. Effect of 500,000 r*

When mixed after irradiation, no clumping of the paramecia occurred; it was not until the third day after irradiation that the mating reaction occurred in which small clumps of two to five individuals were formed. This mild mating reaction then occurred on all subsequent days but did not result in conjugation until a week after irradiation when only a few pairs were present. More pairs formed daily until a maximal number was reached on the thirteenth day. This dosage resulted in an appreciable decrease in numbers of paramecia and also induced great variation in size of specimens.

### *f. Effect of 600,000 r*

No immediate clumping reaction resulted upon mixing but the first appearance of the reaction occurred three days after irradiation. Clumps then consisted of five or less individuals which failed to result in conjugation. After the third day following irradiation clumping occurred daily and did not result in conjugation until on the seventh day when a few conjugants were found. The greatest number appeared on the 12–14th day following irradiation.

Colorless individuals as a result of irradiation at this dosage and at 300,000 *r* are being maintained in permanent culture.

### *g. Effects of 700,000 r to 1,000,000 r*

No mating reaction occurred as a result of these dosages and the animals were seen to move very slowly. Paramecia were unable to survive and all died approximately one to seven hours after irradiation.

### *Effect upon mating reaction and conjugation when only paramecia of one sex type were irradiated and mixed with normal, unirradiated paramecia of opposite sex type*

When paramecia of one sex type are irradiated and mixed with normal, unirradiated paramecia of the opposite sex type, the mating reaction and conjugation occur.

With 300,000 *r* and less, types *C* and *D* of irradiated paramecia produce large clumps when mixed with unirradiated specimens of opposite sex type. These characteristic clumps of the mating reaction are approximately as large as in the controls but require more time to be formed. Similarly, with increased dosages of roentgen units, the lag at which conjugation first appears is lengthened. As an example, those irradiated with 300,000 *r* and mixed with normal specimens of the opposite sex type form clumps of twenty to thirty paramecia in approximately 25 minutes which an hour later develop into clumps of 100 or more specimens. Subsequently, these large clots then break down until 6½ hours after mating only singles are left. On the day following irradiation and mixing, clumping recurs (which in one experiment yielded only one pair firmly joined in conjugation). On the second day clumping recurs which leads into the formation of a number of conjugants but on the third day following irradiation many conjugants are produced after the mating reaction.

After irradiation of one sex type with 400,000 *r* to 700,000 *r*, the paramecia enter into the mating reaction with normal, unirradiated specimens of opposite sex type. The clumps however, are very slow in forming and they are much smaller than in the controls. Thus it requires approximately one hour to form a clump of a dozen or so paramecia when one type is irradiated with 400,000 *r* and there is evidence to show a prolongment of the reaction as noted in the persistence of small clumps to remain long after the animals are mixed. The lag in time in conjugation after the mating reaction increases with the dosage of irradiation until at 700,000 $r$ there is a mating reaction which does not lead into conjugation since the irradiated specimens do not survive this dosage.

### *Result of mixing irradiation-killed paramecia of one sex type with living unirradiated paramecia of opposite sex type*

The author (Wichterman, 1940 p. 437) observed and reported the instance of a union resembling conjugating pairs of a living *Paramecium caudatum* with a dead specimen and raised the question of nuclear behavior occurring in the living member.

Boell and Woodruff (1941) reported a specific mating reaction between living *Paramecium calkinsi* of one mating type and a single dead animal of opposite type in their studies dealing with metabolism of mating types. Later Metz (1947) described the induction of "pseudo selfing" and meiosis in *Paramecium aurelia* by formalin killed animals of opposite mating type. He reported that after mixing, clumps of living and dead paramecia broke down in 60–90 minutes releasing single living animals and also pairs of "pseudo selfing" animals. Pairs joined only at the anterior or "holdfast" region and remained united for approximately five hours. "Pseudo selfing" and conjugation animals appeared to have about the same meiotic time table.

Since it has been shown that dead paramecia of one sex type induce clumping with living members of opposite sex type, experiments were undertaken to discover whether *P. bursaria* killed by irradiation would induce the clumping phenomenon with living unirradiated paramecia of the opposite sex type.

Paramecia were killed with dosages of 700,000—1,000,000 $r$, the speed of mortality depending upon the dosage. It should be remembered that generally before the onset of death, cytolysis occurs in many. After irradiation with 900,000 and 1,000,000 $r$ dead paramecia were mixed with living members of the opposite type. There was no immediate clumping such as is found in the typical mating reaction. However, a striking phenomenon occurred soon after mixing which at first was strongly suggestive of the mating reaction. Large numbers of living specimens of one sex type clumped around intact dead and disintegrating specimens of the opposite sex type. This appears to be a simple food-reaction such as is found when paramecia congregate around a cluster of bacteria since the phenomenon can be demonstrated when living unirradiated specimens are mixed with irradiation-killed paramecia of the same sex type. Soon after complete cytolysis of dead paramecia, large protoplasmic islands or masses became evident which strongly attracted the living specimens. This did not induce or result in the conjugation of living specimens of the one sex type.

### *Effects of irradiated culture fluid upon unirradiated P. bursaria*

Brown, Luck, Sheets and Taylor (1933) studied the action of x-rays upon the ciliate *Euplotes taylori* as well as the effect of irradiated culture fluid alone on the ciliate. They reported that culture fluid when exposed to x-rays did not produce toxic effects or induce death in *Euplotes*. On the other hand Piffault (1939) reported that irradiation of culture fluid in which *Paramecium aurelia* were swimming may produce secondary or toxic effects which should be taken into account in this type of investigation.

Taylor, Thomas and Brown (1933) found that dosages of $46 \times 10^4$ *r* produced death of *Colpidium campylum* within 15 minutes following exposure. They reported that x-radiation with $35 \times 10^4$ *r* of a sterile culture medium of 10 per cent yeast extract, tap and distilled water rendered the medium highly toxic or lethal to the ciliates. Their tests showed hydrogen peroxide in the irradiated water in concentrations above 1 : 100,000. They found that de-oxygenated water following the irradiation gave negative tests for $H_2O_2$ and was not toxic to colpidia. Organic materials such as sheep's blood, agar, gelatin and bacteria, added to the tap water before or after its x-radiation, protected the colpidia against its toxic action. They believed that hydrogen peroxide was in large measure responsible for toxic and lethal effects but suggest that other toxic agents may be produced.

To test the effects of irradiated culture fluid upon unirradiated specimens of *Paramecium bursaria,* experiments were performed in which clear, original culture fluid with paramecia of one sex type (*C*) was irradiated with 1,000,000 *r,* 900,000 *r,* 800,000 *r* and 700,000 *r.* When one cc. of animal-free fluid from these irradiated sets was mixed with three cc. of unirradiated culture fluid containing paramecia of the same or opposite sex type (*D*) no toxic or lethal effects could be distinguished. The same was true when mating type *D* irradiated fluid was added to unirradiated animals of the same sex type or opposite sex type. In daily observations over a period of eight days, there was multiplication of the paramecia with many fission stages evident.

These experiments also disclosed another interesting phenomenon. When paramecia were irradiated at these high dosages and the irradiated animal-free fluid of one sex type was introduced into cultures of unirradiated paramecia of the opposite sex type, clumping of paramecia occurred. The clumps, consisting of a dozen or less paramecia, resembled those characteristic of the mating reaction. The clumps did not lead to selfing or conjugation but broke down into single animals. The fluid lost its ability to induce clumping again when kept for a day and tested. Since the same phenomenon occurred when irradiated, animal-free fluid of one sex type was introduced into cultures of living unirradiated specimens of the same sex type, the clumping is evidently not a mating reaction.

Although not dealing with irradiation studies, Chen (1945) reported that animal-free fluid from a Russian clone of *P. bursaria* induced clumping and conjugation among animals of another mating type even though the latter belonged to a different variety. While the phenomena may be similar to those found in certain algae and other Protozoa, this is probably an example of a "killer" action and not a sex type action.

### *Effects of irradiation upon joined conjugants of P. bursaria*

Experiments were performed in which celluloid boats containing 50 or 100 joined pairs of conjugants were irradiated with 300,000 *r.* Paramecia of opposite mating types and highly reactive were mixed and allowed to form pairs in conjugation. After conjugants were firmly united, pairs were removed with a micropipette and equal numbers then placed into two containers. One container of conjugants represented the controls while the remaining container of conjugants was irradiated 19–20 hours after mating with 300,000 *r.* Both control and ir-

## TABLE II

*Length of time control and irradiated conjugants of P. bursaria remained joined together in conjugation process**

| Hours after mixing opposite mating types | Number of conjugants separated in controls | Number of conjugants remaining in controls | Number of conjugants separated in irradiated set | Number of living conjugants remaining in irradiated set | Remarks |
|---|---|---|---|---|---|
| 20 | 0 | 50 | 0 | 50 | |
| 20½ | 0 | 50 | 0 | 50 | |
| 21½ | 9 | 41 | 0 | 50 | |
| 22¼ | 16 | 25 | 0 | 50 | |
| 22½ | 6 | 19 | 0 | 50 | |
| 23 | 5 | 14 | 0 | 50 | |
| 23½ | 5 | 9 | 0 | 50 | |
| 24 | 3 | 6 | 2 | 48 | |
| 24½ | 6 | 0 | 4 | 44 ← | *Note:* All of controls separated at this time while irradiated conjugants are just beginning to separate. |
| 25 | — | — | 5 | 39 | |
| 25½ | — | — | 0 | 39 | |
| 26 | — | — | 2 | 37 | |
| 26½ | — | — | 2 | 35 | |
| 27½ | — | — | 4 | 31 | |
| 28 | — | — | 2 | 29 | |
| 29 | — | — | 4 | 25 | |
| 29½ | — | — | 1 | 24 | |
| 30 | — | — | 0 | 24 | |
| 30½ | — | — | 0 | 24 | |
| 31 | — | — | 0 | 24 | |
| 31½ | — | — | 0 | 24 | |
| 32 | — | — | 0 | 24 | |
| 32½ | — | — | 0 | 24 | |
| 33 | — | — | 0 | 24 | |
| 33½ | — | — | 0 | 24 | |
| 42½ | — | — | 2 | 22 | |
| 43½ | — | — | 2 | 20 | |
| 44½ | — | — | 0 | 20 | *Note:* Nineteen irradiated pairs of conjugants died during the process. |
| 45½ | — | — | 0 | 20 | |
| 47 | — | — | 1 | 19 | |
| 48 | — | — | 0 | 19 | ↓ |
| 49 | — | — | 0 | 18 | 1 pair died and discarded |
| 53 | — | — | 0 | 18 | |
| 55 | — | — | 0 | 16 | 2 pairs died and discarded |
| 57½ | — | — | 0 | 15 | 1 pair died and discarded |
| 66 | — | — | 0 | 15 | |
| 68 | — | — | 0 | 14 | 1 pair died and discarded |
| 70 | — | — | 0 | 13 | 1 pair died and discarded |
| 75 | — | — | 0 | 13 | |
| 78 | — | — | 0 | 11 | 2 pairs died and discarded |
| 81 | — | — | 0 | 4 | 7 pairs died and discarded |
| 82 | — | — | 0 | 0 | 4 pairs died and discarded |

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* In this experiment there were used 50 pairs of control conjugants and 50 pairs of conjugants irradiated with 300,000 *r* 19–20 hours after mixing members of each sex. All paramecia of one sex type came from one culture while all members of the opposite sex type came from another culture. Temperature during experiment: 24.5–27° C.

radiated conjugants used in an experiment always came from the same cultures, each of course containing the opposite sex types. The temperature ranged between 24.5–27° C. and was the same for each container at any given time. Conjugants of *P. bursaria* remain joined together in the sexual process for approximately 24 hours at 25° C. (Wichterman, 1946, 1948).

A typical experiment consisting of 50 pairs of control conjugants and 50 pairs of irradiated conjugants is shown in Table II. An examination of the table discloses that all of the control conjugants separated into exconjugants 21½–24½ hours after mating It is at about this time however, that the first of the irradiated conjugants begin to separate. It will be seen from the table that irradiated conjugants separated into exconjugants 24–81 hours after mating.

Of the 50 irradiated conjugants, 19 died during the conjugation process. Irradiated conjugants, like normal ones (as is the case with normal and irradiated single specimens), spiral to the left. Irradiated conjugants, especially those joined for approximately 60 hours and longer, become H-shaped. The paramecia separate along their oral surfaces but are held together by a thick conspicuous protoplasmic bridge in the paroral region. This protoplasmic bridge seems comparable to that described and figured by Sonneborn (1947, p. 286) in the conjugation of *P. aurelia.*

## Discussion

In what appears to be an exploratory investigation Bardeen (1906–08) exposed paramecia to x-rays and reported that after a 12 hour exposure, no apparent effect was noted upon the ciliates. He is the first to have observed the effect of x-rays upon conjugants and reported that there was no effect in joined pairs undergoing conjugation or upon their offspring. It is likely that he worked with minimal dosages but this information is lacking from his account.

Schneider (1926), Hance and Clark (1926) and Hance (1931) investigated the effect of x-rays upon vegetative and dividing forms of *Paramecium.* Hance and Clark found the division rate to suffer a slight initial depression lasting two to five days following the exposure. However, the depression was followed by complete recovery. This is in agreement with the author's findings in *Paramecium bursaria* where irradiation was found to temporarily inhibit division. The greater the dosage, the greater the delay in fission. If there is survival of paramecia after exposure to x-rays, the fission rate slowly increases until it is similar to the controls. Indeed, in two of nine isolation cultures in one experiment with *P. bursaria* irradiated with 300,000 *r* and observed daily for 20 days, the division rate was greater than in any of the nine controls.

According to Hance and Clark, dividing specimens showed no different effects than typical vegetative ones when irradiated. They reported that doses repeated at various intervals generally failed to interfere more markedly with the division rate than a single dose. Repeated irradiation caused the paramecia to become slightly swollen without apparent interference with their vitality. According to these investigators, treatments lasting for 10 minutes to three to four hours depressed division and longer or repeated exposures under some conditions raised the reproductive rate.[1]

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[1] According to Hance and Clark (1926) the x-rays were produced at 30 kilovolt peak and 22 milliamperes, filtered through very thin cardboard and applied at a target distance of 25.5 cm. Such rays produced about $6 \times 10^{12}$ pairs of ions per gm. per second in air.

In this regard, Back (1939) irradiated *Paramecium caudatum* to ⅔–⅚ of the lethal dose and found that division of the ciliates could be suspended for several weeks. Accordingly, he considered this lack of division a type of injury inflicted upon the organism.

With *Colpidium colpoda,* a ciliate closely related to *Paramecium,* Crowther (1926) found that a considerable exposure to x-rays produced little visible alteration either in the appearance or motion of the organisms. In an investigation upon the same species, C. Lloyd Claff (personal communication) came to the same conclusion.

Crowther observed that with a given sub-lethal exposure, the ciliates became perceptibly accelerated with rapid and excited appearing behavior followed by a zig-zag motion. When removed at this stage, the majority of ciliates survived. Lethal effects were noted when the forward progression stopped, vacuoles became distended and with the formation of a bubble of clear substance which was extruded from the body, followed by death. It was found that the protoplasm of irradiated *Colpidium* appeared less transparent and distinctly more granular than in normal specimens.

With *P. bursaria,* somewhat similar effects were noted with sub-lethal dosages of 100,000 *r* in that the paramecia became more active in their swimming behavior. Also similar blebs or vacuoles of a clear, structureless substance were formed upon the pellicle of *P. bursaria* after longer irradiation and active cyclosis was considerably decreased or even stopped.

In an inconclusive paper Baldwin (1920) reported the combined action of x-rays and of vital stains upon paramecia but more recently Halberstaedter and Back (1943) observed the effect of the combined action of colchicine and x-rays and colchicine alone upon *Paramecium caudatum.* The concentrations of crystalline colchicine in the basic culture media were 0.0005 per cent, 0.00025 per cent and 0.000125 per cent with the x-ray intensity at the distance of the irradiated object being 80,000 r./m. In colchicine combined with x-rays, the paramecia were maintained for 48 hours in the colchicine solution then transferred to normal culture media for irradiation. They showed that the lethal dose for normal paramecia fluctuated between 200,000 and 700,000 *r,* the half-value doses lying between 300,000 and 250,000 *r.* Their experiments demonstrated that the immediate lethal dose is considerably smaller—about 50 per cent—for paramecia which received treatment earlier with colchicine.

Brown, Luck, Sheets, and Taylor (1933) studied the action of x-rays upon another ciliate, *Euplotes taylori,* in culture fluid and on culture fluid alone. They found that culture fluid exposed to x-rays did not induce death or produce toxic effects on *Euplotes.* The minimal lethal dose to kill the protozoon was found to be 2110 roentgen units per second but much less exposure was sufficient to kill the associated bacteria in the culture fluid. Irradiated bacteria were found to be unsatisfactory as a source of food for *Euplotes.* This difference in resistance to x-ray exposure made it easily possible to sterilize this ciliate. Their important observation adds another method for the sterilization of protozoa and it is very likely that many other species can be sterilized in the same manner.

They found that when *Euplotes* was irradiated for 100–220 seconds there occurred a complete cessation of ciliary activity in many of the organisms, the percentage so affected increasing with the length of exposure. Hours after irradiation such organisms which were seemingly dead regained ciliary movement later.

Later Back and Halberstaeder (1945) made a compreshensive study of the effect of x-rays upon *P. caudatum* and its culture fluid. They found that doses of 100,000 *r* were without visible effects. This is in agreement with the author's findings with *P. bursaria* at this dosage and the paramecia completely recover although they appear excited immediately after irradiation. However, with increased irradiation of paramecia Back and Halberstaeder noted visible alteration of the manner of movement. The change was noted when one-half of the immediate lethal dose was applied. Cilia failed to behave normally and the paramecia swam irregularly backward and forward. With greater dosage, movement ceased and cytolysis occurred. They reported that complete cessation of movement served as an indication of the impending death of the organism and define the immediate lethal dose for *P. caudatum* as that dose which produced complete cessation of motility within 10–15 minutes after irradiation. When 1,000,000 *r* were applied to the culture media alone, no toxic or secondary effect was observed when paramecia were placed in the irradiated fluid. This experiment was repeated by the present author with *P. bursaria* who also found no toxic effect upon the ciliates which is in sharp contrast to the reports of Piffault (1939). It occurs to the author that Piffault may have first killed the bacteria in his irradiated paramecia cultures thereby depriving the ciliates of their source of food. In the experiments reported in the present paper, bacteria known to support growth in other colorless races, were added to the irradiated paramecia. Perhaps the secondary effect reported by Piffault is nothing more than starvation of the paramecia.

Back and Halberstaeder (1945) performed experiments to determine the difference in roentgen-ray susceptibility between immediate (family) and remote (clone) descendents of a single individual. The family represents four to eight descendents of a single organism. Applications of 200,000 *r* caused death in a small percentage of the irradiated population. With increase in the dosage, this percentage rises. At 600,000 *r*, almost all the irradiated specimens died. All survivors of this treatment died after the dose was increased to 700,000 *r*. The dose which produces a 50 per cent mortality rate lies between 350,000 *r* and 400,000 *r*.

They found that when paramecia were irradiated in the same drop, members of a clone died at different roentgen-ray doses. However, under the same conditions, members of a family always died at the same dose.

Concerning tolerance to irradiation of clones within a species, the present work on *Paramecium bursaria* disclosed a difference in the mating types which may only be racial. It was found that members of mating type *C* are more susceptible to x-rays than those of opposite mating type *D*.

It is noteworthy to point out that no one has attempted to make a cytological study of the nuclei or nuclear behavior in irradiated paramecia. Such a study is likely to yield interesting and valuable results.

## SUMMARY

1. Individuals of opposite mating type of *Paramecium bursaria* were irradiated with roentgen rays at dosages ranging from 100,000 *r* to 1,000,000 *r* in steps of 100,000 *r*.

2. After 100,000 *r*, locomotion of the paramecia became markedly accelerated but with higher dosages locomotion was retarded. None was able to survive dosages of 700,000 *r* or more.

3. Clones of *P. bursaria* have been established in the laboratory from specimens irradiated with 300,000 *r*–600,000 *r*. One result of such irradiation is the destruction of the contained green symbiotic zoöchlorellae living with in the paramecia. A method is now available for the production of colorless clones of *P. bursaria*.

4. Colorless *P. bursaria* as produced by irradiation have the same sex type as before irradiation. Such specimens readily mate and conjugate with members of the opposite sex type.

5. Members of one mating type are more susceptible to x-rays than those of opposite mating type. This may in reality be only a racial, not a sexual difference.

6. Paramecia of opposite sex types will, when irradiated and mixed, enter into the mating reaction. Clumps thus formed are smaller than in controls and the time taken to form them is greater. The lag in time between conjugation and the mating reaction is dependent upon the dosage.

7. Irradiated conjugants remain joined together in the sexual process much longer than those of the controls.

8. Irradiation inhibits division but the effect is only temporary. After a period of time (depending upon the dosage) survivors have a division rate similar to control specimens.

9. Irradiated culture fluid has no toxic or lethal effect upon unirradiated specimens of *P. bursaria.*

10. Irradiation-killed specimens of one sex type do not mate or conjugate with living, unirradiated members of the opposite sex type nor does it result in selfing.

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# STUDIES ON MARINE BRYOZOA. II. BARENTSIA LAXA KIRKPATRICK 1890

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## Introduction

The purpose of the present study is (1) to report the occurrence of *Barentsia laxa* Kirkpatrick, 1890, an Entoproct of the Family Pedicellinidae, in the waters of Woods Hole, Massachusetts, thus adding to the list of species already reported from that area; (2) to call attention to the interesting association between this species and *Cliona*-supporting *Venus* shells; and (3) to give a more detailed, more completely illustrated account of the morphology and reproduction of this species than has been available up to now.

Most grateful acknowledgment is due the Marine Biological Laboratory of Woods Hole, Massachusetts, whose Supply Department and Collecting Crew dredged the specimens, and also to the Laboratory for working facilities without which this study would not have been possible.

## History

*Barentsia laxa* was originally described by Kirkpatrick (1890, p. 624, Pl. XVII, Fig. 6) from a station between Orman's Reef and Brothers Island (Gaba), Torres Straits, which are between Australia and New Guinea. It was next reported from the Bay of Bima and east of Pajunga Island, Kwandang Bay, the Malay Archipelago, by Harmer (1915, p. 32, Pl. II, Figs. 10–11). Livingstone (1927, p. 69) reported it from the coastal waters of Queensland, the Great Barrier Reef and outlying islands of Australia. Hastings (1932, p. 401) briefly noted that *Barentsia laxa* had been previously reported from Australian waters. Marcus (1938, pp. 10–11, Pl. II, Fig. 3; 1939, pp. 212–214, 275, Pl. 20, Fig. 51) reported it from the Bay of Santos, Brasil, and Osburn (1944, pp 10–11, Fig. 3) reported it from Chincoteague Bay, Chesapeake Bay and from a station 15 miles east of Great Point , Nantucket Island (in the Atlantic Ocean). The species thus has been reported only a few times and from widely separated areas.

*Barentsia laxa* was found on various substrates by the aforementioned workers: (1) on *Flustra cribriformis* by Kirkpatrick; (2) on a coral, *Porites,* by Harmer; (3) on coral, shells, sea weeds and other Bryozoa by Marcus (1938, p. 10); and (4) on hydroid stems, oyster shells and over *Anguinella palmata* by Osburn. The exact dates of collection or the breeding time of *Barentsia laxa* have not been recorded by the earlier workers although some observed larvae in the colonies. Such data would be useful to those doing experimental work on the species.

The following data are based entirely on the *Barentsia laxa* specimens collected at Woods Hole.

## Collection Data

The present *Barentsia laxa* was collected in Great Harbor, Woods Hole, Massachusetts, at a depth of approximately 30 to 50 feet, on the following dates: VII-25-1945, VIII-10-1945, VII-25-1946, VIII-8-1946, VII-25-1947, VIII-7-1947 and VIII-28-1947.

A number of empty *Venus* shells encrusted with yellow, boring *Cliona* sponge, dredged by the Collecting Crew for use in the Invertebrate Zoology Course of the M.B.L., were transferred to aquaria which were supplied with running sea water. On these shells were tufts of *Barentsia laxa,* half an inch tall and from one-half to one inch in extent. The colonies were a tan or grey color and of rather soft texture. Some of the *Venus* shells were badly riddled and covered with sponge. The *Barentsia* was more abundant on these than on the cleaner, less diseased shells. It grew as well directly on the sponge surface itself, that is, on the patches of sponge covering or eating through the shell, as it did on the shell proper. It was much easier to scrape the *Barentsia* from the sponge surface than from the shell surface; thus the stolons as well as the rest of the colony could be obtained intact. Although numerous *Pecten, Crepidula,* other Pelecypod and Gastropod shells were dredged from Great Harbor, they seemed to be much less utilized as substrates by *Barentsia laxa* than were the *Cliona*-infested *Venus* shells. However, some fine extensive colonies were found on a large cinder clinker, so *B. laxa* is not found exclusively on shells or other animals.

*Barentsia laxa* colonies are quite hardy and very easy to keep in the laboratory without any particular care or feeding other than to have fresh running sea water flowing constantly into their aquarium. Colonies collected in July 1947 were still alive by September 4, 1947, although many of the individuals had lost their heads and were in the process of regenerating new ones. Some of the slender stalks were headless but still alive and writhing about.

Free-swimming larvae and embryos in various stages of development were obtainable throughout the time of study (July 25 to September 4) and very probably considerably earlier and later.

## General Morphology

*Barentsia laxa* is the third *Barentsia* species to be reported from the immediate vicinity of Woods Hole. The other two, *B. discreta* and *B. major,* were reported by Osburn (1912, pp. 13–14).

*Barentsia laxa* forms colonies of numerous pin-like individuals crowded together to make a soft furry mass or tuft sometimes up to one-half inch in height. The colonies grow somewhat more frequently on the inner protected surface of an empty *Cliona*-infested *Venus* shell near the hinge and umbo than elsewhere. Each zoid consists of three major parts: the calyx (head), stalk, and swollen base. Each zoid is connected with neighboring zoids by stolons of varying length. Measurements of these various parts are given in Table I and the parts are illustrated on Plate I.

TABLE I

*Comparison of the Woods Hole Barentsia laxa specimens with those from other localities*

| | Woods Hole specimens | | | | Other *Barentsia laxa* specimens | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of readings | Maximum | Minimum | Average | Harmer (1915, pp. 32–33) | Kirkpatrick (1890, p. 624) | Marcus (1938, p. 10) | Osburn (1944, pp. 10–11) |
| Calyx length, with tentacles rolled in | 21 | .518 mm. | .216 mm. | .300 mm. | .480 mm. | .4–.5 mm. | .400 mm. | .55–.78 mm. |
| Calyx width, with tentacles rolled in | 22 | .432 mm. | .144 mm. | .240 mm. | .365 mm. | .35–.4 mm. | .330 mm. | .40–.52 mm. |
| Stalk length | 60 | 9.045 mm. | .374 mm. | 4.810 mm. | 2.500 mm. | | 1.600 mm. | |
| Stalk width, near calyx | 20 | .137 mm. | .029 mm. | .078 mm. | .080 mm. | | .075 mm. | |
| Stalk width, near basal enlargement | 20 | .048 mm. | .029 mm. | .038 mm. | .032 mm. | | .040 mm. | |
| Musclium length, from septum to base | 24 | .576 mm. | .216 mm. | .359 mm. | .480 mm. | .25 mm. | .350 mm. | .20–.35 mm. |
| Musclium width | 24 | .245 mm. | .086 mm. | .134 mm. | .190 mm. | .16 mm. | .150 mm. | .12–.20 mm. |
| Stolen length | 12 | 1.008 mm. | .072 mm. | .464 mm. | | | | |
| Stolon width | 22 | .058 mm. | .029 mm. | .039 mm. | | | .045 mm. | |
| Total length (calyx, stalk and musclium lengths added) | 2 | 9.867 mm. | 1.024 mm. | | 3.460 mm. | 3.00 mm. | 2.350 mm. | 6.5 mm. maximum |
| Tentacle number | 50 | 23 | 13 | 17 | about 20 | | about 20 | |

## CALYX

The calyx (Figs. 23, 24, 26, 30, 32, 33) bears the tentacles, digestive tract, reproductive system, larvae, nephridia, ganglia and nerves, and is at the tip of the slender stalk which connects it with the musclar base.

The anterior, esophageal side of the calyx is flattened. The posterior, intestinal side is more curved (gibbose). Its wall is relatively thin and transparent. Internal organs are readily visible. The lophophore may be contracted, with tentacles bent inward, as shown in Figures 19, 20, 24 and 33, or may be expanded with tentacles spread outward, as in Figures 5, 16 and 22. A strong circular muscle layer, whose fibers are indicated in Figures 19, 22, 24, 30 and 33, is present in the calyx rim, making possible the closure of the calyx rim or velum over the rolled in tentacles. The observed tentacle number ranged from 13 to 23.

The inner surfaces of the tentacles and the floor of the atrium are ciliated (Fig. 16).

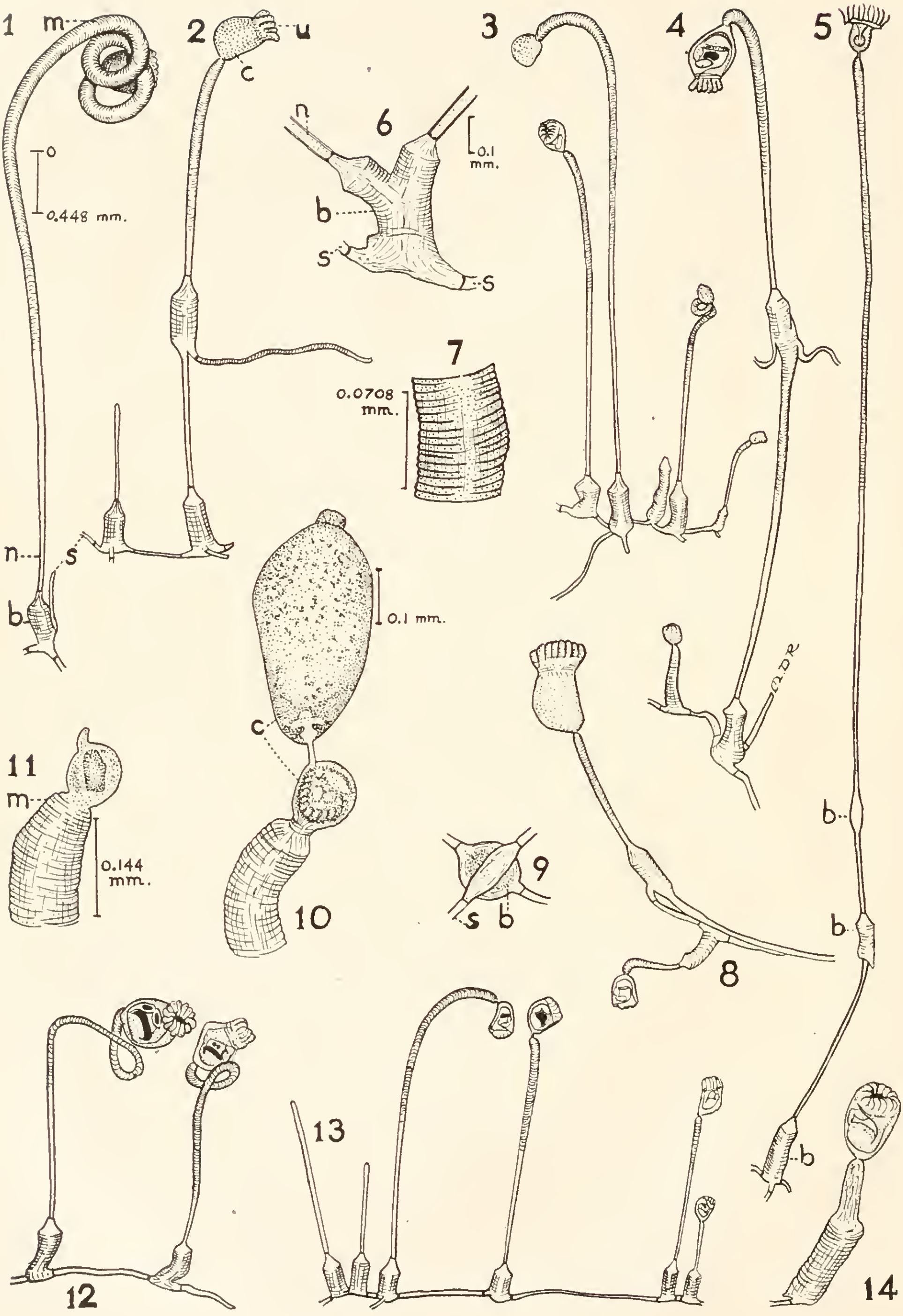
Into the atrium open the mouth, anus, nephridia and reproductive organs.

The most conspicuous set of organs within the calyx is the digestive tract. Its walls are very clearly defined. The tract consists of mouth, pharynx, esophagus, esophageal valve, stomach (whose upper wall is thickened into a so-called "liver"), intestine, rectum and anus. The pharynx is wider than the esophagus which is a fairly long vertical tapering tube (Figs. 15, 24). Beyond the esophageal valve is the horizontal, much enlarged sac-like stomach whose wall varies in thickness in different areas and in different physiological states. The "liver" differs in color from the rest of the tract. It is a deep yellow, sometimes brownish color while the rest of the tract is white or gray. Large cells stud its surface (Figs. 15, 17, 18, 24). The sphincter between stomach and intestine may at times be so relaxed (Fig. 26) that the boundary between the two organs is hard to define. This is true when a continuous mass of food begins to revolve in both organs at the same time, or when food begins to pass from the stomach into the intestine. At other times, the boundary between stomach and intestine is very easy to see (Fig. 23). The intestine leads upward, somewhat at an angle, toward the horizontally placed rectum. The rectum is partially hidden by the lophophore, atrium, tentacles and gonads. When full it may cause an elevation of the atrial floor (Fig. 42).

All the chambers of the digestive tract are ciliated although the cilia may be more prominent in some parts of the gut than in others. A very conspicuous tuft of cilia is found at the entrance to the stomach, near the esophageal valve (Fig. 24). Shorter cilia occur in other parts of the stomach also (roof, sides and floor) but the abovementioned tuft is the most conspicuous. Cilia line the intestine and rectum also.

*Barentsia laxa* feeds on Protozoa, Bacteria, other small organisms and debris. The food seems to get entangled in a strand of mucus in the stomach and the whole mass may rotate for a time in one direction (Figs. 23, 26), then reverse and rotate in the opposite direction, because of the action of the cilia lining the tract. The lower end of the mucus strand is attached for a time to the stomach wall. Fecal pellets rotate in either direction in the intestine and rectum as does the food mass in the stomach.

PLATE I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## Explanation of Plate I

Figures 2, 8 and 9 are free-hand drawings; the remainder on this plate were drawn with the aid of a camera lucida. The scale alongside Figure 1 applies to Figures 1, 3, 4, 5, 12 and 13. The scale alongside Figure 6 applies to that figure alone. The scale alongside Figure 7 applies to that figure alone. The scale alongside Figure 10 applies to that figure alone. The scale alongside Figure 11 applies to Figures 11 and 14. All Figures were drawn from living specimens of *Barentsia laxa.*

Figure 1. *Barentsia laxa* zoid showing great flexibility of the upper part (pedicel) of the stalk. The pedicel is shown in a double coil, almost hiding the head. The lower part of the stalk is less flexible.

Figure 2. A colony of two individuals, one of which has lost its head and the other provided with an "elbow" or additional intercalated musclium from which arises an annulated stalk or stolon, an unusual condition. These jointed individuals are very scarce in this species. A septum has not yet formed immediately below the intercalated musclium.

Figure 3. A small colony of four zoids of differing age and length connected by short stolons. Most colonies were of this type, i.e., without additional musclia intercalated between the basal musclium and the calyx.

Figure 4. Shows the great contrast in length between a young and an old zoid. The large older zoid is unusual in that it is "elbowed" or provided with an intercalated musclium from which arise two stolons. This is not so frequent a condition as that of Figure 3.

Figure 5. An unusually long zoid with two fully formed musclia (*b*) and the beginning of a third. The middle musclium was separated from the stalk by a septum above and below. The uppermost enlargement apparently was not yet far enough developed to have septa. The calyx on this specimen had about 17 tentacles.

Figure 6. An unusual basal musclium, fused at the lower end and split at the upper end.

Figure 7. Portion of a pedicel showing the closeness of the annulations. A few of the longitudinal muscle fibers are indicated.

Figure 8. Another unusual condition, that of an intercalated musclium giving rise to a stolon which continues to grow along the parent stalk, using the latter as a substratum.

Figure 9. View of a basal musclium as seen from the basal or attached surface, showing the relative position and point of origin of four stolons.

Figure 10. An old degenerated calyx is attached by a thin strip of tissue to a newly developing calyx which is being differentiated from the tip of the pedicel.

Figure 11. A pedicel is topped by a young, regenerating calyx. The old calyx has been lost, leaving the small protoplasmic projection from the upper left surface of the young calyx as the only remnant of the connection between old and new calyces. The contents of the young calyx of Figure 11 are not as far along in development as the calyx contents of Figure 10, or more properly, the zoid of Figure 11 lost its old calyx sooner than did the zoid of Figure 10. This mode of calyx loss and regeneration is not uncommon, several zoids having been found in various stages of degeneration and regeneration.

Figure 12. Two zoids showing the characteristic coiling and ceaseless twisting about of the upper part of the flexible *Barentsia laxa* stalks.

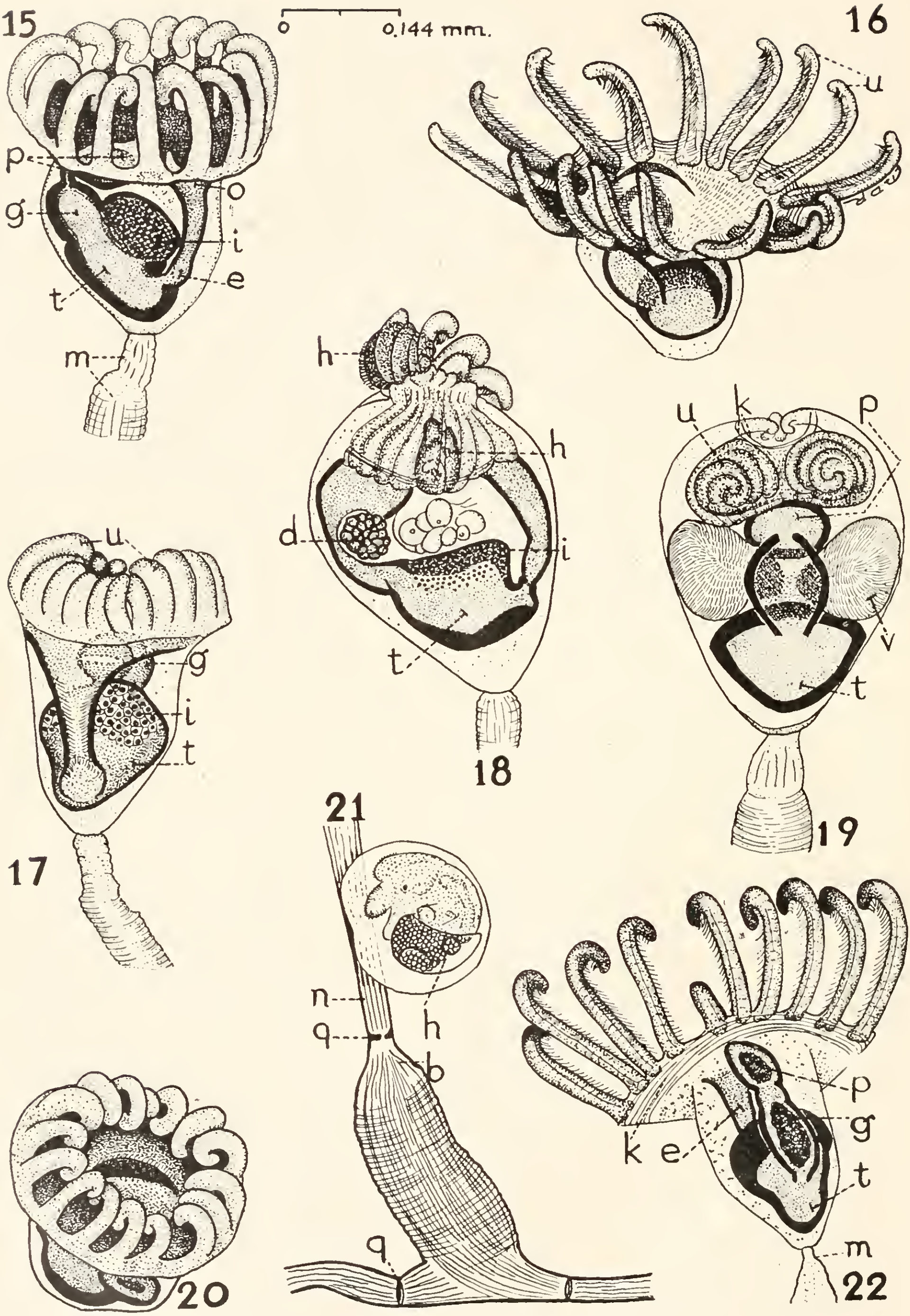
Figure 13. A typical small colony showing four whole individuals and two which had lost their calyces. Two of the stolons are quite long while the others are very short.

Figure 14. A very young zoid. No septum has yet formed to divide the stalk from the musclium.

## Abbreviations Used on Plates

ao—Aboral organ
a—Anus
b—Basal enlargement or musclium
c—Calyx
d—Embryo
e—Esophagus
f—Flagella
g—Intestine
h—Larva
i—Liver
j—Mouth
k—Muscle fibers
l—Ovary
m—Pedicel
n—Peduncle
o—Pharynx
p—Rectum
q—Septum
r—Sperms
s—Stolon
t—Stomach
u—Tentacles
v—Testis
w—Velum

PLATE II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The body cavity or space between body wall and gut is quite empty-looking except for very sparsely arranged parenchymal cells whose meshes are exceedingly large (Figs. 24, 31), and whose few protoplasmic processes are very slender and attenuated. When living specimens were treated with a few drops of a 1 per cent aqueous Neutral Red solution the cytoplasm of some of the parenchyma cells took up the red color intensely while the nuclei remained light in color.

A pair of very small nephridia (Fig. 25) occur just in front of the testes, ovaries and back of the pharynx, just beneath the artial floor. They are very tiny and but for the intermittent flicker of their internal flagella would not have been noticed.

The nervous sytem is difficult to distinguish in living specimens except for a ganglion which is present behind the esophagus, above the liver and partially hidden by the ovarial tissue.

The musculature was not studied in much detail. Muscle fibers were observed leading to the esophagus (Fig. 24), in the body wall, lophophore, tentacles, digestive tract wall, stalk and muscular base.

Calyces were found either with (Figs. 18, 19, 23, 24) or without (Figs. 17, 22) reproductive organs. The latter condition was not uncommon, especially in very young zoids. There were calyces with male organs and calyces with female organs but hermaphrodites were not observed in the Woods Hole specimens. Further

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### Explanation of Plate II

All figures on this plate were drawn with the aid of a camera lucida and to the same magnification, the scale of which is shown at the top of the plate. All are figures of *Barentsia laxa,* drawn from living specimens.

Figure 15. View of the right side of a young calyx, showing an empty gut but a very well developed "liver" (*i,* very coarsely stippled area). The zoid was without any apparent gonads as yet. Drawn VIII–16–1947.

Figure 16. View of opened lophophore showing the tentacle arrangement around its rim, the vestibule and mouth. The most heavily darkened crescent-shaped structure below the lophophore (in the calyx cup) is the "liver." It is not so large proportionately as in the preceding figure. The floor of the vestibule or atrium is slightly raised in the anal region.

Figure 17. Calyx as viewed from the esophageal side. The funnel-shaped pharynx-esophagus lies along the more flattened side of the calyx. The "cellular" formation directly behind the esophagus is the "liver." Drawn VIII–15–1947.

Figure 18. View of the right side of a female individual showing an ovary, an embryo (*d*), and two larvae (*h*). One larva is passing upward between the bases of the clustered tentacles. The other larva is ready for release and beginning to work its way out from among the tentacle tips. The thickness of the stomach wall sometimes varies and such variation is here shown. The lophophore is tightly contracted, temporarily. Drawn VIII–31–1947.

Figure 19. View of a male zoid from the intestinal side. The tentacles are folded temporarily into the vestibule or atrium and the lophophore rim is tightly contracted above them. The digestive tract is outlined heavily in black. The testes (*v*) are the two large masses above the stomach, one on either side of the intestine. Drawn VIII–31–1947.

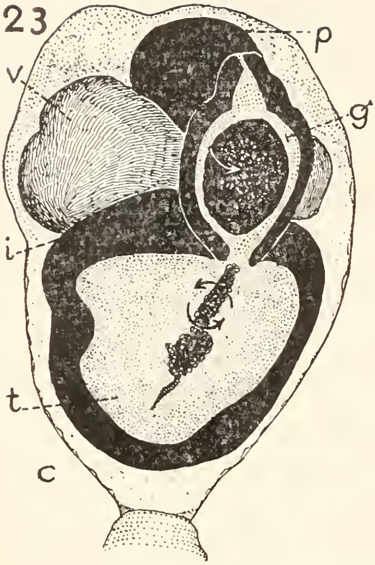
Figure 20. View of tentacles curving inward, preparatory to the "closing" of the calyx.

Figure 21. Detail of swollen base or musclium, showing the direction of muscle fibers. On some specimens, as on this one, mollusk larvae (*h*) may be attached to the stalk or base. Drawn VIII–31–1947.

Figure 22. View of calyx from intestinal side. The digestive tract is heavily outlined in black. Two fecal pellets are shown, one in rectum, one in intestine. The circular musculature of the lophophore is indicated. No gonads were present in this zoid at this time. Drawn on VIII–17–1947.

Plate III

23



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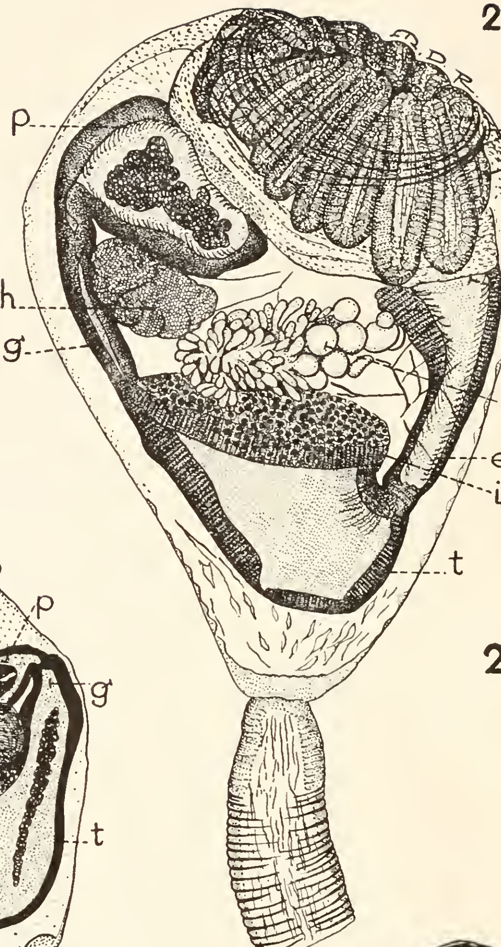
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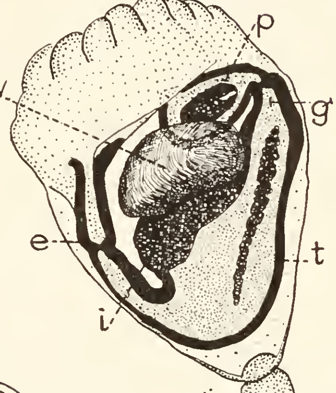

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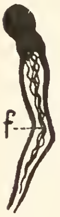

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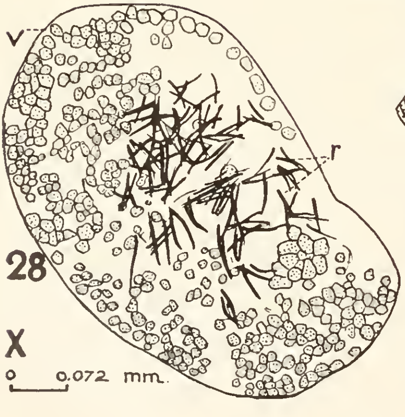

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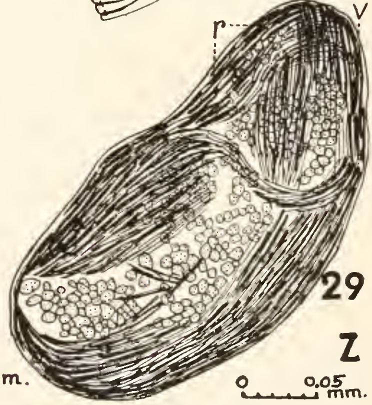

X
0 0.072 mm.

Y
0 0.03 mm.

Z
0 0.05 mm.

study is needed to determine if hermaphrodite specimens may occur in *Barentsia laxa.*

The male gonads are two large dense grey masses, one on either side in the space between liver, intestine and rectum (Figs. 19, 23, 26, 28, 29). Numerous sperms may be seen as fine thin lines within the testes (Figs. 27, 29). When mature the sperms show considerable flickering activity within the testes. Testes which have voided much of the sperm material are rather empty-looking (Fig. 28).

The ovaries are pictured in different stages of development in Figures 18 and 24. They are located above the liver, around the ganglion and slightly forward of the testis position.

Developing embryos seem to be pushed closer to the intestine, posteriorly to the ovary (Fig. 30).

## STALK

The stalks bearing the calyces are long, slender, flexible and generally separated from the basal enlargement by a partial septum. Their length varies with age. The young ones are very short (Fig. 14), the old ones are very long (Figs. 1, 3, 12, 13) and only occasionally show a tendency to become secondarily jointed or articulated along their extent (Figs. 2, 4, 5, 8). This secondary articulation was not previously known to occur in *Barentsia laxa.* The longest stalk measured slightly over 9 mm. in length. The Woods Hole *B. laxa* specimens were generally somewhat longer than those reported from other localities. Table I gives more complete data on these measurements.

The stalk terminology is somewhat confusing. Hincks (1888, p. 226) called the stiff, lower, proximal part of a *Barentsia major* stalk the "pedicel" and the

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### EXPLANATION OF PLATE III

All figures on this plate are of *Barentsia laxa* and were drawn with the aid of a camera lucida, from living material. Scale X applies to Figures 23, 26. Scale Y applies to Figures 25, 27, 28, 29. Scale Z applies to Figure 24.

FIGURE 23. A male zoid as seen from the posterior or intestinal side. The gut is heavily outlined in black. The two testes (*v*) are shown on either side of the intestine. The direction of rotation of the food mass in stomach and intestine is indicated by arrows. Reversal of direction follows after a short interval. Drawn on VIII–30–1947.

FIGURE 24. Enlarged view of the right side of a zoid which possesses an ovary, developing eggs and embryos. The liver is especially prominent in this specimen at time of sketching (VIII–7–1947). Cilia are diagrammatically shown in the pharynx, esophagus, stomach and rectum. They are also present in the intestine (here shown empty and collapsed) and in other parts of the stomach but are not here shown in all the areas in which they should occur. A mesh of a few strands of muscle fibers can be seen attaching to the esophagus. An embryonic mass is back of the ovary and directly below the rectum.

FIGURE 25. A nephridium.

FIGURE 26. A male zoid seen from the left side. A long cord of mucus, to which is attached food material, is seen extending from the stomach into the intestine. The opening between the stomach and intestine was so large at this particular instant that their mutual boundary line was difficult to locate.

FIGURE 27. Diagram of a spermatozoon. Drawn VIII–30–1947.

FIGURE 28. A testis with relatively few spermatozoa in it. Drawn on VIII–30–1947.

FIGURE 29. A testis with many mature sperms in it, close to the limiting membrane. The sperms at this stage of development are a flickering mass of fine lines. Drawn on VIII–31–1947.

## Plate IV

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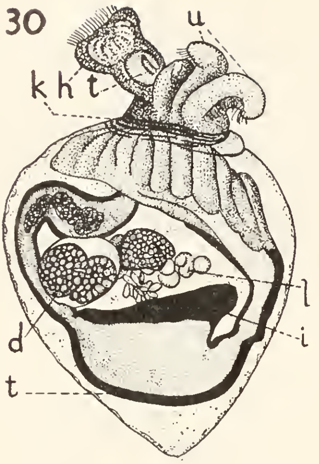

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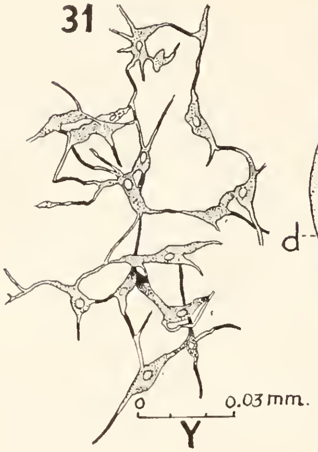

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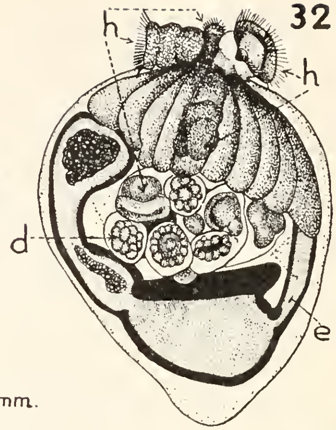

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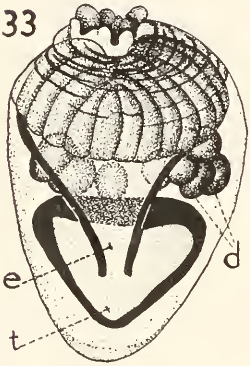

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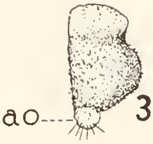

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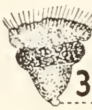

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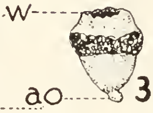

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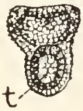

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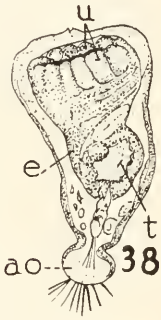

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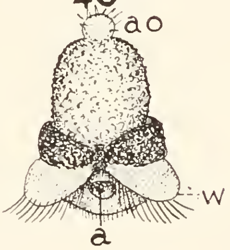

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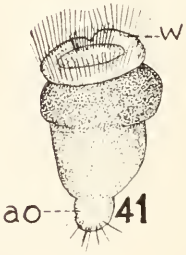

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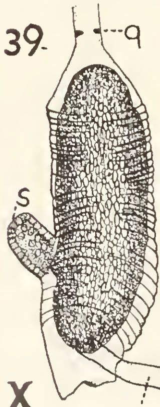
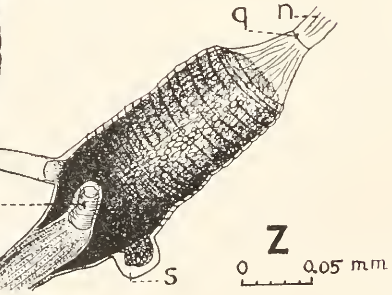
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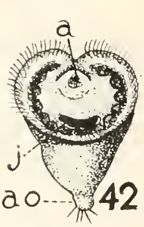

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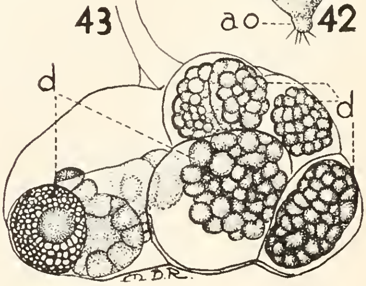

flexible, distal part nearest the head the "peduncle." Jullien and Calvet (1903, p. 27) reversed the names for the parts, calling the stalk segment nearest the head the "pedicellium" (pedicel) and the stalk segment nearest the enlarged base the "pediculium" (peduncle). The basal enlargement they labelled the "musclium basal." Jullien and Calvet's terminology seems to be more acceptable than Hincks' in this case.

The pedicel of *Barentsia laxa* is soft, flexible, annulated (Figs. 1, 7, 10, 12, 24). It is slightly and suddenly narrowed at its junction with the calyx (Figs. 19, 26). It also tapers, but very gradually, toward the less flexible lower portion of the stalk (peduncle). The pedicel is so flexible that it may coil very tightly into one

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## EXPLANATION OF PLATE IV

All figures are of *Barentsia laxa* and are drawn from living specimens. Figures 40 and 42 were drawn free-hand. The remainder were drawn with the aid of a camera lucida. Scale X applies to Figures 30, 32, 33, 34, 35, 36, 37 and 39. Scale Y applies to Figure 31. Scale Z applies to Figures 38, 41 and 43.

FIGURE 30. View of the right side of a female calyx carrying embryos in various stages of development. One larva is being released from between the tentacle tips. The parent digestive tract is heavily outlined in black.

FIGURE 31. A patch of the extremely diffuse parenchymal tissue which partially fills the body cavity or space between body wall and gut. It is quite inconspicuous in the calyx.

FIGURE 32. A female calyx as seen from the right side, carrying a number of embryos in various stages of development. Twelve embryos or larvae are visible from this side. Three larvae are clustered at the tips of the tentacles, ready for release. Two more are shown in the vestibule among the tentacles (near the tentacle bases), on their way upward and out. There are a number more enclosed in delicate transparent membranes between the liver and the tentacle bases. Drawn on IX–1–1947.

FIGURE 33. A female calyx seen from the esophageal side, showing nine multicellular embryonic masses whose cell detail is not shown. Collected on VIII–28–1947 and drawn three days later.

FIGURE 34. Left side of a young larva which had been released from the parent calyx an hour earlier. The flat and gibbose surfaces are very distinct in this view. The larval body covering is characteristically roughened.

FIGURE 35. Larva with velum or calyx edge expanded.

FIGURE 36. Same larva as shown in Figure 35 but with velum slightly contracted or bent inward. Drawn on IX–1–1947.

FIGURE 37. Recently released larva showing the stomach and the slightly roughened external surface. Aboral organ retracted into calyx at the moment. Drawn on VIII–31–1947.

FIGURE 38. Larva just at the instant of release, before it has had time to swim. Its digestive tract is well defined. The stomach, esophagus and vestibule are especially noticeable. Tentacle buds are distinguishable. Drawn on VIII–31–1947.

FIGURE 39. Basal enlargements of two zoids. The musclium at right shows four stolons; the one at left shows a stolon and a stolon bud. These basal enlargements are lined and partly filled with heavily granular cells which give them an opaque look. Drawn on VIII–18–1947.

FIGURE 40. A released larva seen from anal view, with aboral organ at top, ciliated velum expanded and anus visible near the indentation of the velum. Anal opening is functional at this stage. Drawn on IX–1–1947.

FIGURE 41. Another view of the same larva but with its calyx rim or velum (*w*) more contracted.

FIGURE 42. A recently released larva seen from the top, showing a fecal pellet, mouth and the dark, heavily granular tentacular area. The tentacles are not yet very distinct in this specimen.

FIGURE 43. A brood chamber with several larvae in different stages of development. The larval blastomeres are heavily granular. Some larvae have already been expelled, judging from the emptiness of the chamber. Drawn on IX–1–1947.

or two coils (Figs. 1, 2). This constant coiling and uncoiling is very annoying when one is trying to study anatomical details in living specimens. Sometimes, the zoids of the whole colony may be actively writhing and bumping heads. The annulations are very close together (Fig. 7, 24) and may or may not extend all the way down to the basal enlargement, depending upon the age of the zoid. Longitudinal muscle fibers occur inside the stalk.

### BASAL ENLARGEMENT

The basal enlargement or musclium is very distinct, even in young zoids (Figs. 3, 14, 21). It is cylindrical, tapering sharply and suddenly at its distal end to merge with the peduncle. Proximally, the musclium gives rise to one or more slender stolons which connect it with neighboring zoids (Fig. 9). It is closely annulated, very muscular and capable of some movement (Figs. 12, 21, 39). Some specimens are fairly transparent (Fig. 21). Others are quite opaque (Fig. 39) because of the numerous heavily granular cells within them. In the latter state the musculature appears to be outside the layers of opaque cells.

The cuticle of older basal enlargements thickens and turns yellow but the annulations are still visible.

Occasionaly one sees stalks of very long or old individuals swollen here and there (Fig. 5) and a basal musclium (b) making its appearance along the stalk. Some of these joints develop so completely that they sprout stolons (Figs. 2, 4, 8). This jointed condition is relatively rare, however.

### STOLON

Stolons are slender, flexible, cylindrical, muscular connections between basal enlargements of various zoids (Figs. 2, 3, 4, 8, 13). They vary in length from 0.07 to 1 mm. They creep along the substratum over or along each other if crowded. Figure 8 shows a rather unusual condition, where a stolon has bent back to grow along a stalk from which it originated. If several arise from a musclium they do so at slightly different levels (Fig. 9). If only two stolons are present they are generally in a straight line across the slightly narrowed bottom of the musclium (Fig. 21). If four stolons are present, they are usually or approximately at right angles to each other, and in two lines, one line passing slightly above the other (Fig. 9).

Septa occur along the stolons near the musclium but have not been observed elsewhere along the stolon (Figs. 9, 21).

### CALYX REGENERATION

Colonies which are kept in the laboratory for any length of time begin to lose their heads, possibly because of inadequate food suply. The internal organs of the calyx degenerate and some of the calyces become quite empty (Fig. 10). Under these old degenerating calyces may develop a bud from the tip of the pedicel. This bud, which will form the new head, is separated from the old head by a thin protoplasmic strip (Fig. 10) which becomes thinner or narrower as time goes on, until the old head breaks away from the stalk at that point. For a short time the protoplasmic remnant projects from the new developing calyx (Fig. 11), then is obliterated by the growth of the calyx and the gradual healing of the break. New heads may therefore be found on old stalks. It would be interesting to see just how many times regeneration of a calyx could take place on the same stalk and how fast is the regeneration rate.

Stalks may remain viable and capable of writhing movement for a number of days without their heads.

When living specimens were stained with a 1 per cent aqueous Neutral Red solution the growing tip of the pedicel stained an intense pink in contrast to the pale pink color of the rest of the stalk and stolon.

## Larvae

Developing *Barentsia laxa* embryos were found in considerable numbers from the last week in July to the first week in September, at Woods Hole. They very likely occur earlier and later than the dates indicated but the writer did not have the opportunity to collect them earlier or later than these dates.

Larvae and embryos in various stages of development were found simultaneously in the same calyx (Figs. 30, 32). The largest number of larvae observed in a parent zoid at any one time was nine but this very likely is not the maximum number. The larvae occupy the space above the liver, between the pharynx, intestine and rectum. They find their way out through a channel opening into the atrial floor and leave the body in front of the anal opening Several larvae may be released in succession (Fig. 32) within a few seconds or minutes of each other.

The larval digestive tract seems to be functioning just before the release of the larva from the parent. A fecal mass spins about in the gut of some of the about-to-be-shed larvae. Some larvae get rid of a fecal pellet just at the moment of their release from the parent calyx. The lophophore of the parent may be contracted or relaxed during the release of the larva. Within a few seconds after release, the larval body surface becomes roughened and debris-laden. The larvae swim about very actively for quite a time, some for at least an hour, others longer. During this interval the digestive tract becomes more clearly defined although the larval body wall is still fairly opaque. The shape of the larva becomes more gibbose, as in the adult calyx. The larval body is very flexible. Larvae undergo some bodily contortions of shortening and lengthening. They can retract their aboral organ, then extrude it again, may temporarily attach to the substratum by means of it, slide along the substratum on it, then let go and swim about actively again.

Anatomically, *Barentsia laxa* embryos show great similarity to embryos or larvae of *Pedicellina echinata,* judging from Hatschek's beautifully illustrated account of the development of the latter species.

Measurements of a recently released *Barentsia laxa* larva, with aboral organ retracted into body cavity, are as follows: length 0.144 mm., width at widest part 0.086 mm.

## Miscellaneous Data

Some of the other animals found in association with the Woods Hole *Barentsia laxa* are mollusk larvae (veligers), Vorticellids, *Pseudofolliculina*(?) and the following Bryozoa: *Aeverrillia armata, Bowerbankia imbricata* and *Pedicellina* *cernua.* The veliger larvae are deposited in capsules along the *Barentsia* stalks, muscular bases or stolons (Fig. 21). They rotate or squirm about actively within their transparent globular capsules. The protozoa are found attached to the calyces and among the stolons. The Bryozoa grow over and among the stolons and enlarged bases of the *Barentsia* zoids.

Incidentally, this is the first report of the occurrence of *Bowerbankia imbricata* from the Woods Hole area. It has been observed on a number of occasions in the vicinity by the author.

## SUMMARY

1. This is the first report of *Barentsia laxa* and *Bowerbankia imbricata* from the immediate Woods Hole region.

2. A much more complete description, numerous measurements and more detailed illustrations of *Barentsia laxa* than have been previously available have been here presented.

3. Larval behavior, time of appearance and place of development for larvae of *Barentsia laxa* have been noted, to aid those who wish to make a further study of *B. laxa* embryology.

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# THE ROLE OF SLIME FILM IN THE ATTACHMENT OF FOULING ORGANISMS

MILTON A. MILLER,[1] J. C. RAPEAN[2] AND W. FOREST WHEDON[3]

## INTRODUCTION

The attachment of larvae of fouling organisms to submerged surfaces is influenced, in varying degree, by many factors. Among these, are several dependent upon particular properties of surfaces including the tendency to acquire slime or primary films when exposed in sea water. Sometimes called microfouling, these films usually constitute the first attachment to submerged surfaces; they are formed by marine bacteria, diatoms, and other microorganisms together with adherent organic and inorganic detritus (Herpin and Duliscouet, 1938; Waksman, Ketchum, Phelps, and Weiss, 1941; Whedon, 1937; ZoBell and Allen, 1935; ZoBell, 1938, 1939a, 1939b, and 1939c). Some types of paint coatings are decomposed by marine bacteria (Darsie, 1943; Renn, 1942), and the decomposition products may also contribute to the film. Depending largely on the type of surface on which it forms, the film may be sparse or copious; silty, granular or gelatinous (Adamson, 1937).

The effect of slime film on attachment of fouling organisms has long been a controversial issue. Some investigators have suggested that the presence of a slime film on a surface may be prerequisite to the attachment process (Angst, 1923; Whedon, 1937–1939). Others, however, have observed that larvae of various sedentary organisms may attach to cleaned or newly submerged surfaces before any visible film is formed (Hilen, 1923; Whedon, 1939; Miller and Cupp, 1942; Miller, 1946). Hence, a slime film is apparently not necessary for attachment of larvae. Nevertheless, it may facilitate their attachment. ZoBell (1939c) and Phelps (1942), for example, found that slime film on glass surfaces favors attachment of barnacle cyprids. ZoBell (1939b) suggests that the slime film might promote attachment in several ways: (1) by enmeshing the free-swimming larvae or otherwise mechanically facilitating their attachment; (2) by discoloring glazed or bright surfaces; (3) by serving as a source of food; (4) by protecting the attached organisms from the toxic constitutents of poisonous paints; (5) by increasing the alkalinity of the film-surface interface, thereby favoring the deposition of calcareous cements; or (6) by influencing the potential of the surface.

The practical effect of slime film on the antifouling properties of toxic paint surfaces has also been a debated question. Following Bray (1923), several authors have claimed a relationship between the amount of slime film formed on a surface and its antifouling properties. Bray first observed that those surfaces which developed the heaviest films ultimately became the most heavily fouled. Later, however, Bray (1924) noted that plastic types of paints appeared to form a slime film more readily than others, and that these remained free of fouling growths for longer periods. He suggested that the slime film (scum) itself might act as an antifouling coating by "mechanical hindrance to attachment," by retaining the toxic agent, or by maintaining a "higher degree of acidity than the sea water." Bray found a correlation of 0.65 ± 0.09 (P.E.) between the degree of fouling on plates exposed for six months and the acidity of their slime films, but he was uncertain as to the reason for it. Adamson (*op. cit.*) also pointed out that gelatinous films which form on some types of surfaces seem to discourage fouling attachment, but silty or granular films permit it.

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These early suggestions of a relationship between slime film and fouling led to extensive investigations on the subject, notably by Whedon at Scripps Oceanographic Institution, La Jolla, California (Whedon, 1937–1941); by Whedon and staff at the Naval Biological Laboratory at San Diego, California (Whedon, *et al.,* 1942 and 1943); and by investigators at Woods Hole, Massachusetts (Ferry and Davidson, 1942; Ketchum and Davidson, 1942; Waksman, Ketchum, Phelps, and Weiss, 1941; Waksman, Ketchum, and Weiss, 1941; Waksman, Ketchum, and Davidson, 1942). Most of these studies were designed to test specific points in the so-called film theory (see below). They contributed much valuable information on the properties of slime film and of the underlying paint surface. More importantly, they led to the demonstration that the rate of loss of toxic ions from the paint surface (leaching rate) is the primary factor in prevention of fouling (Ketchum, *et al.,* 1945; Miller, 1946). As a result, emphasis in research on prevention of marine fouling shifted to physico-chemical investigations on mechanisms controlling the leaching rate (Ferry and Carritt, 1946; Ferry and Ketchum, 1946; Ketchum, Ferry, and Burns, 1946).

According to the slime film theory, the long-term prevention of serious fouling on painted surfaces is dependent upon the development of an adequate slime film. Such a film supposedly becomes the antifouling surface by taking up and concentrating toxic materials released by the underlying paint. Presumably, the toxic substances are either adsorbed by, or combined with components of the slime, or precipitated therein. Furthermore, microorganisms in the film are assumed to aid the release of toxic from the paint in one or both of two ways: (1) by lowering the pH at the paint surface thus favoring the solution of cuprous oxide or other metallic salts commonly used as toxic ingredients of antifouling paints; and (2) by decomposing the paint matrix, thus accelerating the penetration of sea water into the paint with consequent increase in the rate of solution of its toxic components.

The mode of action of the slime film in the prevention of fouling has not been clearly stated, although several suggestions have been advanced. The most plausible one is that toxic substances, accumulated by the film, might inhibit establishment of fouling by releasing toxic ions. Contact toxicity has also been suggested. Physical effects of the film that have been mentioned include mechanical hindrance to attachment of larvae and exfoliation. The term "exfoliation" implies that the surface film or pieces of it may be sloughed off, especially when subjected to swift currents or to rapid movements of the surface through the water, and that any fouling organisms which were attached only to the slime film would also be shed in the process.

There is some more or less presumptive evidence in favor of the slime film theory. (1) Toxic substances such as copper may be much more concentrated in the slime film than in the underlying paint itself (Whedon, *et al.,* 1942–1943). (2) Renn (*op. cit.*) determined that the acidity of water adjacent to slime-coated surface might be increased by as much as 0.6 pH unit as a result of $CO_2$ production by microorganisms in the film. Such a change would increase several fold the rate of solution of toxic salts, and thus the slime film might contribute to the antifouling properties of a surface. (3) Darsie (*op. cit.*) demonstrated that slime film bacteria decompose or utilize w.w. rosin and paraffin which are matrix ingredients of certain good antifouling paints, but they do not affect ester gum, an ingredient of some inferior paints. His experiments thus indicate that bacterial erosion of the matrix may be an important mechanism in the release of toxic materials from a paint, and, in this indirect way, the slime film might contribute to the effectiveness of a toxic paint.

In spite of these several ways in which the slime film might accelerate the release of toxic materials, repeated tests have shown that the leaching rate of filmed surfaces is generally *less* than that of identical surfaces from which the slime film has been removed (Whedon, *et al.,* 1943). In extreme instances, the slime film over a toxic surface may reduce its leaching rate by half or a third, but the average depression in leaching rate would probably not exceed 20 per cent. Occasionally, filmed surfaces were found which had higher leaching rates than their non-filmed counterparts, but these differences were generally slight and within the experimental error. When leaching rates are well above the minimum adequate level to prevent fouling, the observed differences would be inconsequential except insofar as they might influence the effective life of a paint. Maintained over long periods of time, however, a depressed leaching rate would tend to conserve the toxic content, while long-continued acceleration of the leaching rate obviously would hasten its depletion. In the case of so-called borderline paints with leaching rates near the minimum adequate level, however, the presence of a slime film might be beneficial, detrimental, or neither depending on whether it increases, decreases, or has no effect on the rate of toxic loss. Available evidence indicates that the slime film (and other factors such as light, color, and surface texture) become increasingly important as the leaching rate decreases.

The direct approach to the question of the relationship of primary film to fouling would be to compare attachments of organisms to various surfaces with and without a coating of slime film. Critical experiments of this sort are difficult or impossible to conduct under field conditions for two basic reasons: (1) a visible film forms on submerged surfaces in a relatively short time and usually long before macroscopic fouling organisms appear, thus making it impossible to maintain a slime-free surface during the period of time required for such comparative field tests; and (2) it is hard to control adequately the many variables involved in the attachment of fouling in nature. Quantitative laboratory tests using larvae of a bryozoan, *Bugula neritina* L., were evolved which eliminated the above-mentioned difficulties. Since *Bugula* larvae settle within a few hours after they are liberated from the parent colonies, they can be used in critical experiments comparing the effects of presence and absence of slime film on larval attachment.

The primary purpose of the experiments reported in this paper was to determine the effect of slime film on the attachment of *Bugula* larvae to various toxic and non-toxic surfaces. It is assumed that the conclusions drawn from these laboratory studies would have general application since the tests were designed, as far as possible, to simulate natural conditions, and since it has been shown that results of *Bugla* larval tests correlate nicely with data obtained in field trials.

## METHODS

The biological experiments feature two sets of conditions: (1) tests in which the free-swimming *Bugula* larvae were offered panels either completely coated with slime film or with the slime film entirely removed, so that the larvae were allowed no choice as between filmed and clean surfaces for attachment; and (2) tests using panels from half of which the slime was removed, so that attaching larvae had an equal chance of settling on filmed or clean surfaces. The former are referred to hereafter as "no-choice tests," the latter as "choice tests."

The general procedure was the same for both types of test. Small, 1 × 3 inch panels, appropriately prepared (see below), were suspended in separate glass jars, each containing 700 cc. of fresh sea water. Immediately thereafter, a known number of active *Bugula* larvae was added. Tests were started in midmorning using larvae that had been liberated from parental colonies earlier in the day. Counts of attached larvae were made late in the afternoon of the same day when it was evident that no more larvae would settle. (Those that do not affix themselves to the panels either die, or occasionally attach to film or debris at the surface of the water, or rarely to the smooth glass sides of the container.) During the tests, the water was aerated and slightly agitated by a fine stream of air bubbles delivered at the base of the jar by a tapered glass tube.

Test panels were 1 × 3 inch steel or ground glass plates and were prepared as follows. The steel panels were first prime-coated with an anti-corrosive paint and then covered with the various experimental formulations. The paints selected for study are grouped in four series (Table I, A to D) and differ from each other in two important respects, toxicity and matrix composition. From preliminary work, both of these variables were suspected of having a significant effect on the slime film, and hence attempt was made to test paints differing widely in these respects. The results here reported are typical of a much larger number of tests.

Gradations in toxicity were obtained in two ways: by changing the proportions of cuprous oxide in paints within a series, and by the use of different matrices. A non-toxic panel was included in each series, and, as an additional control, ground glass slides were used.

Of the four matrices, three were hot plastic (Table I, Series A, C, and D) and one was cold plastic (Series B). The matrices in the three hot plastic series all contain paraffin but differ in the resinous ingredient, which, as will be seen, seems to be a critical component. It will be noted that the matrices of Series A and C differ only in that the latter substitutes ester gum for the w.w. rosin content of Series A. In Series D, ester gum is substituted for only one-fourth the w.w. rosin content of Series A.

Prior to testing, the panels were "seasoned" by immersion for a period of four to ten weeks in large jars of aerated sea water which was changed at frequent intervals, usually every other day. During the seasoning period the panels acquired ample coatings of slime film.

Just before or sometimes after the larval tests, chemical tests were made which included leaching rate determinations and analyses of the copper content of the slime film. Leaching rate tests were made using the method described in detail by Ketchum and co-workers (1945). Slime films were carefully removed from the test panels with a rubber squeegee and dried at 110° C. for analysis. After pulverizing in a mortar, the dry films were examined microscopically for the presence of eroded paint matrix, and all samples thus contaminated were discarded. Copper was determined using the sodium diethyl dithiocarbamate method (Ketchum, *et al., op. cit.*) after the organic matter was destroyed with nitric and perchloric acids. The subsequent preparation of the panels differs for the choice and no-choice attachment tests, and will be described separately.

*a. No-choice attachment tests.* Just before testing, the slime film was wiped entirely from one member of each duplicate pair of panels, but left intact on the other. These panels will be designated the "defilmed" and "filmed" panels, respectively. Since each panel is submerged in a separate test jar, the larvae must attach either to filmed or to defilmed surfaces, or not at all.

As an index of toxicity, the percentage of attachment was calculated for each panel by dividing the number of larvae attached by the original number placed in the test jar. These percentages were compared to determine whether there were any statistically significant differences in toxicity between the filmed and defilmed surfaces for each paint. All other conditions being equal, any significant differences in frequency of larval attachment must be attributed to an influence of slime film.

*b. Choice attachment tests.* For these, the slime film was removed from half the surface area of each panel. On one side, the slime film was removed only from the center half, leaving it intact on the two end quarters; whereas, on the opposite side, only the end quarters were wiped leaving the center half covered with film (Plate I, Fig. 2). This pattern of wiping was designed to nullify the effect of extraneous variables, such as light and position, that might influence the larvae in their choice of an attachment site. When these panels were suspended in test jars (a separate one in each), the larvae thus had an equal chance of settling on filmed or defilmed areas.

At the end of the test, the number of larvae attached to each area was counted. If only chance was involved in their attachment, equal numbers of larvae would be expected to settle on filmed and on cleaned areas. Any significant deviation from a 1-to-1 ratio of attachment between filmed and defilmed areas would therefore indicate an effect of slime film.

## Results and Discussion

The experimental data are given in Tables I and II, but for convenience, the results on attachment of *Bugula* larvae to toxic and non-toxic surfaces will be discussed separately.

### *Effect of slime film on attachment of Bugula larvae to non-toxic surfaces.*

To establish a norm of behavior of the *Bugula* larvae in the two attachment tests, it will first be necessary to consider their reactions to non-toxic surfaces, with and without a coating of slime film. As a general rule, the larvae attached abundantly and in about equal numbers to both filmed and defilmed non-toxic painted panels when they were given no choice of conditions (Table I, Nos. 6. 11, 14, and 20; also, Plate I, Fig. 1). If significant differences occurred, they were generally small and inconsistent, being sometimes in favor of the filmed, sometimes the defilmed panels. On ground glass panels (No. 21), however, the slime film apparently did facilitate larval affixation as significantly greater numbers of larvae attached on the filmed panels than on the defilmed panels. It is pertinent

TABLE I

*Attachment of Bugula larvae to toxic and non-toxic panels.* (1) *No-choice test:* panels either entirely coated with a slime film or with slime film completely removed; attaching larvae thus given no choice of surface conditions. (2) *Choice test:* slime film removed from half of each panel so that larvae had an equal choice of attaching to filmed or to defilmed surfaces.

| Series and type of paint | Paint No. | Per cent $Cu_2O$ in dry paint | Weeks of seasoning prior to testing | (1) No-choice test | | | (2) Choice test | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Percentage attachment ±S.E. | | Type of panel with greater attachment[1] | Number of larvae attached to: | | Preferred condition of surface[1] |
| | | | | Filmed panels | Defilmed panels | | Filmed half | Defilmed half | |
| | | % | | % | % | | | | |
| *Series A*<br>Hot plastic (matrix composed of about equal parts of w.w. rosin and paraffin) | 1 | 37.0 | 4 | 1±0.9 | 3±1.7 | — | 4 | 1 | — |
| | | | 10 | 4±1.6 | 2±1.1 | — | | | |
| | 2 | 32.0 | 4 | 6±1.7 | 4±1.4 | — | 8 | 36 | Defilmed |
| | | | 10 | 23±5.3 | 15±4.4 | — | 13 | 9 | — |
| | 3 | 26.0 | 4 | 5±1.6 | 93±1.7 | Defilmed | 3 | 148 | Defilmed |
| | | | 10 | 5±3.3 | 13±6.3 | — | 2 | 23 | Defilmed |
| | 4 | 19.0 | 4 | 7±1.7 | 79±1.7 | Defilmed | 1 | 102 | Defilmed |
| | | | 10 | 79±3.5 | 72±5.0 | — | 7 | 51 | Defilmed |
| | 5 | 10.6 | 4 | 84±2.6 | 98±1.1 | Defilmed | 14 | 77 | Defilmed |
| | | | 10 | 86±4.4 | 95±2.6 | — | 39 | 117 | Defilmed |
| | 6 | 0 | 4 | 85±3.5 | 95±1.4 | Defilmed | 72 | 19 | Filmed |
| | | (control) | 4 | 76±4.3 | 48±4.9 | Filmed | 95 | 48 | Filmed |
| | | | 10 | 100 | 100 | — | 79 | 5 | Filmed |
| *Series B*<br>Cold plastic (series based on Navy Dept. Specification F 143 E) | 7 | 36.0 | 4 | 3±1.4 | 4±1.7 | — | 3 | 2 | — |
| | | | 10 | 0 | 3±2.4 | — | 5 | 2 | — |
| | 8 | 30.0 | 4 | 2±1.4 | 8±2.8 | — | 3 | 5 | — |
| | | | 10 | 5±3.0 | 1±1.4 | — | 2 | 1 | — |
| | 9 | 19.2 | 4 | 13±3.1 | 17±3.6 | — | 52 | 7 | Filmed |
| | | | 10 | 7±2.4 | 10±4.5 | — | 8 | 2 | — |
| | 10 | 9.3 | 4 | 62±4.6 | 81±3.3 | Defilmed | 90 | 30 | Filmed |
| | | | 10 | 87±3.6 | 73±6.8 | — | 25 | 3 | Filmed |
| | 11 | 0 | 4 | 76±3.9 | 39±4.8 | Filmed | 90 | 6 | Filmed |
| | | (control) | 10 | 78±7.4 | 98±1.4 | Defilmed | 56 | 6 | Filmed |

TABLE I—*Continued*

| Series and type of paint | Paint No. | Per cent $Cu_2O$ in dry paint | Weeks of seasoning prior to testing | (1) No-choice test | | | (2) Choice test | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Percentage attachment ±S.E. | | Type of panel with greater attachment[1] | Number of larvae attached to: | | Preferred condition of surface[1] |
| | | | | Filmed panels | Defilmed panels | | Filmed half | Defilmed half | |
| | | % | | % | % | | | | |
| *Series C*<br>Hot plastic (matrix composed of equal parts of ester gum and paraffin) | 12 | 37.0 | 4 | 13±3.4 | 6±2.4 | — | 13 | 8 | — |
| | 13 | 32.0 | 4 | 61±4.7 | 11±3.2 | Filmed | 65 | 11 | Filmed |
| | | | 10 | 27±4.7 | 1±1.1 | Filmed | | | |
| | 14 | 0 | 4 | 99±0.9 | 97±1.4 | — | 165 | 77 | Filmed |
| | | (control) | 6 | 77±5.9 | 81±5.5 | — | 34 | 5 | Filmed |
| | | | 6 | 82±3.8 | 85±3.6 | — | 39 | 21 | Filmed |
| | | | 10 | 85±5.0 | 90±4.2 | — | | | |
| *Series D*<br>Hot plastic (matrix as in Series A but with ester gum substituted for 25% of the w.w. rosin) | 15 | 40.0 | 4 | 9±3.1 | 3±1.5 | — | 7 | 2 | — |
| | 16 | 32.0 | 4 | 32±4.4 | 17±3.7 | Filmed | 79 | 19 | Filmed |
| | 17 | 24.0 | 4 | 49±5.0 | 29±3.9 | Filmed | 94 | 49 | Filmed |
| | 18 | 16.0 | 4 | 11±2.7 | 57±4.0 | Defilmed | 48 | 27 | Filmed |
| | 19 | 8.0 | 4 | 81±3.5 | 81±3.3 | — | 107 | 64 | Filmed |
| | 20 | 0<br>(control) | 4 | 99±0.9 | 97±1.4 | — | 165 | 77 | Filmed |
| *Series E*<br>Ground glass | 21 | 0 | 6 | 91±4.0 | 61±6.9 | Filmed | 45 | 4 | Filmed |
| | | (control) | 6 | 91±2.8 | 52±5.0 | Filmed | 65 | 23 | Filmed |

[1] Only statistically significant differences or preferences in larval attachments are indicated; dashes signify no significant difference. In several instances, the number of larvae attached was too small to give significance to the differences between filmed and defilmed panels in the no-choice test, or to the deviations from the 1:1 ratio expected by chance in the choice test.

to mention that the larvae do not attach to the smooth glass sides of the container unless it is smudged or filmed.

When offered a choice between filmed and defilmed areas, however, the larvae consistently and definitely preferred to attach to the slime film, regardless of the composition of the underlying paint (Plate I, Fig. 2). The average ratio of numbers of larvae attached to filmed and defilmed areas of the non-toxic panels was nearly 4-to-1; in every instance, there was a significant deviation from the 1-to-1 ratio that would be expected on the basis of chance.

Whether the larvae actually "choose" attachment sites is an open question.

TABLE II

*Chemical tests on paints after four or ten weeks of seasoning in the laboratory*

| Series and paint number (see Table I) | Leaching rate* $\mu g.\ Cu^{++}/cm.^2/day$ |  | Copper in slime film (% of organic matter) |
|---|---|---|---|
|  | Filmed | Defilmed |  |
| A–1 | 22.4–28.1 | 31.7–24.1 | 7.37 |
| –2 | 21.1–19.4 | 28.0–23.7 | 8.22 |
| –3 | 16.0–14.0 | 19.4–14.0 | 4.50 |
| –4 | 19.0–13.5 | 20.9–11.5 | 4.61 |
| –5 | 5.1– 6.9 | 5.6– 5.8 | 1.41 |
| –6 (control) | —— | —— | —— |
| B–7 | 18.9–14.3 | 29.7–14.1 | 6.73 |
| –8 | 19.7–14.1 | 29.2–13.3 | 6.40 |
| –9 | 14.6–14.1 | 16.8–13.0 | 8.39 |
| –10 | 4.2– 3.5 | 3.6– 3.8 | 1.44 |
| –11 (control) | —— | —— | —— |
| C–12 | 23.8 | 26.0 | 4.75 |
| –13 | 7.0 | 14.3 | 1.83 |
| –14 (control) | —— | —— | —— |
| D–15 | 24.9 | 26.0 | 2.92 |
| –16 | 16.4 | 20.5 | 2.95 |
| –17 | 16.3 | 16.7 | 2.45 |
| –18 | 10.8 | 10.8 | 1.88 |
| –19 | 6.2 | 5.7 | 0.83 |
| –20 (control) | —— | —— | —— |
| E–21 (control) | —— | —— | —— |

* The first values given for Series A and B are leaching rates obtained after the panels had been seasoned four weeks; the second are those obtained after a 10-week seasoning period.

They seemingly explore a relatively sizeable area for considerable periods of time (sometimes several hours) before settling. Sensory cilia at the aboral end of the larvae are probably employed in selecting a favorable location for attachment. Whatever the mechanism involved, the data clearly show that slime-coated surfaces are preferred by the larvae, but they will attach equally abundantly if such are not available. These findings need to be kept in mind in interpreting larval reactions to filmed *versus* clean toxic surfaces.

*Effect of slime film on attachment of Bugula larvae to toxic surfaces.*

The attachment test data (Table I) show that the slime film may influence the antifouling properties of a toxic surface in one of three ways: (1) it may have no

FIG. 1

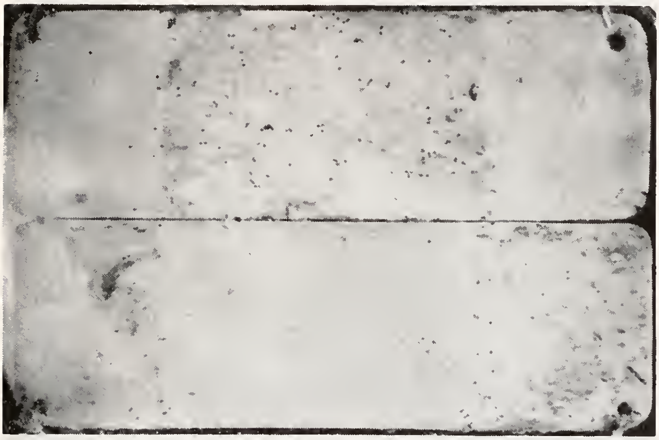

FIG. 2

Plate I. Attachment of *Bugula* larvae (black dots) to non-toxic test panels (Table I, No. 6) with and without coatings of slime film.

Figure 1 shows about equal attachment of larvae to a completely filmed panel (upper), and to its duplicate (lower) from which the slime film was completely removed. The larvae were given no choice between the two types of surface in this test (no-choice test).

Figure 2 shows that *Bugula* larvae prefer to attach to film-coated surfaces when given a choice between filmed and defilmed areas of non-toxic panels. The two panels shown represent the front and back sides of a single panel as used in the choice tests. The slime film was removed from the two end quarters of the front side (represented by the upper panel) and the center half of the back side (represented by the lower panel), so that half the surface area of each panel was cleaned and the larvae thus had an equal chance of settling on a filmed or a slime-free surface.

apparent effect on surface toxicity; (2) it may render the surface less repellent to larvae; or (3) in special instances, it may make the surface more repellent. These effects will now be considered in greater detail.

When offered only film-coated or only clean panels for attachment (no-choice tests), the *Bugula* larvae settled about equally on either in most tests (Table I). Painted surfaces with high leaching rates and high copper content (Table I: Nos. 1, 2, 7, 8, 12, and 15; and Fig. 1) permitted only a few larvae to attach, regardless of the presence or absence of a slime film. Slime films formed on these paints had relatively high copper content, as would be expected. As leaching rates decreased, especially below the "minimum adequate level" of 10 $\mu$g. of copper per square centimeter per day (Ketchum, *et al.*, 1945), more larvae were able to attach, and sometimes there were significant differences in the numbers attached to filmed and defilmed panels (Table I: Nos. 3, 4, 5, 10, 16, 17, and 18). These differences occurred only in tests on panels that were seasoned four weeks and disappeared when they were seasoned an additional six weeks. In general, the results of the no-choice tests show that the leaching rate is the primary factor regulating the attachment of *Bugula* larvae (Fig. 1), and that the slime film is a secondary or modifying factor which becomes more important as surface toxicity decreases.

When given a choice between filmed and defilmed surfaces, however, the *Bugula* larvae show marked preferences in attachment (Table I and Plate II) except in tests on the most toxic paints in each series. These paints allowed attachment of but few larvae (as in the no-choice tests), and no preference was apparent. In all other tests, significantly greater members of larvae attached either to the filmed or to the defilmed areas. The results of the two attachment tests are quite consistent; if larvae attached in greater numbers to filmed panels in the no-choice tests, they generally preferred the filmed areas of panels in choice tests and *vice-versa*.

In interpreting the results of the choice attachment tests, one must remember that the larvae normally prefer to attach to the film-coated areas of non-toxic panels. Therefore, their preference for filmed areas of toxic panels does not necessarily mean that the defilmed areas are more toxic. But preferential attachment to clean or defilmed areas, which is the reverse of the normal tendency, can only mean that the film-coated surfaces, in such instances, are more repellent to the larvae. Some of these differences presumably were too slight to show in the no-choice attachment tests. Corresponding and significant differences in larval attachment obtained in both the choice and no-choice tests, however, may be considered more important.

The presence of a slime film did not seem to improve the repellent properties of toxic surfaces except in one group of paints, namely Series A. Here the larvae consistently preferred the defilmed areas of toxic panels in the choice tests and attached in greater numbers to the defilmed toxic panels in the no-choice tests (whenever any differences were exhibited). In toxic paints of all other series, the larvae preferred the filmed surfaces in the choice tests, and sometimes attached in greater numbers to filmed panels in the no-choice tests.

The rather clear-cut difference between Series A and all other series in the direction of larval attachment seems to be associated with the composition of the paint matrix, more particularly with its resinous ingredient. The combination of w.w. rosin with cuprous oxide, as in Series A paints, seemingly produces surface slime

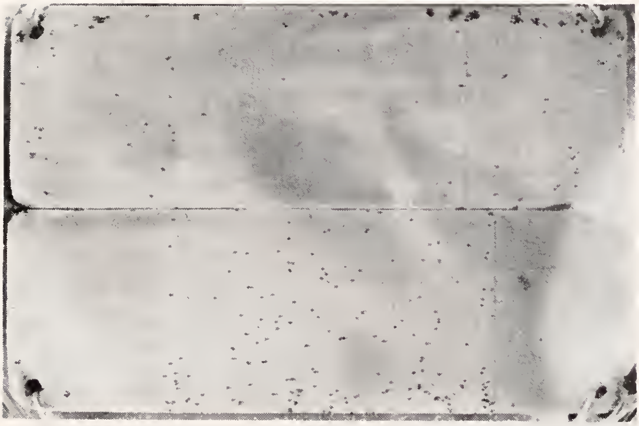

FIG. 1

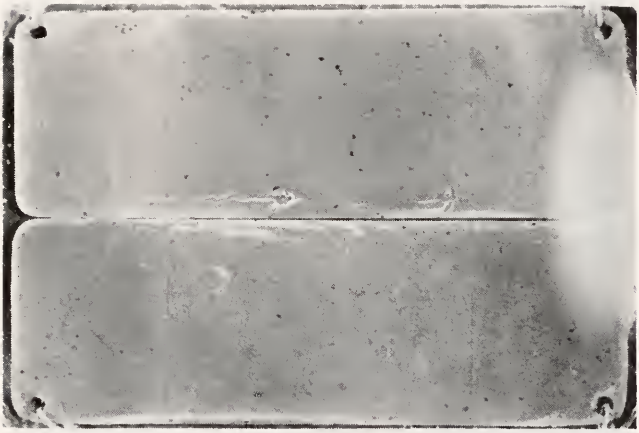

FIG. 2

Plate II. Reversal of preference of *Bugula* larvae in choice attachment tests on two different kinds of copper-painted panels.

Figure 1 shows a larval preference for slime-coated areas (end sections of upper panel and center sections of lower) of a slightly toxic cold plastic paint (Table I, No. 9). This does not necessarily indicate that the defilmed areas are more repellent since the larvae also prefer the filmed areas of control panels in these tests.

Figure 2 shows a preference of larvae for defilmed areas (center section of upper panel and end section of lower) of a slightly toxic hot plastic paint (Table I, No. 5). This indicates that here the slime-coated areas are more repellent, especially since the filmed areas are preferred in the control for this series of paints.

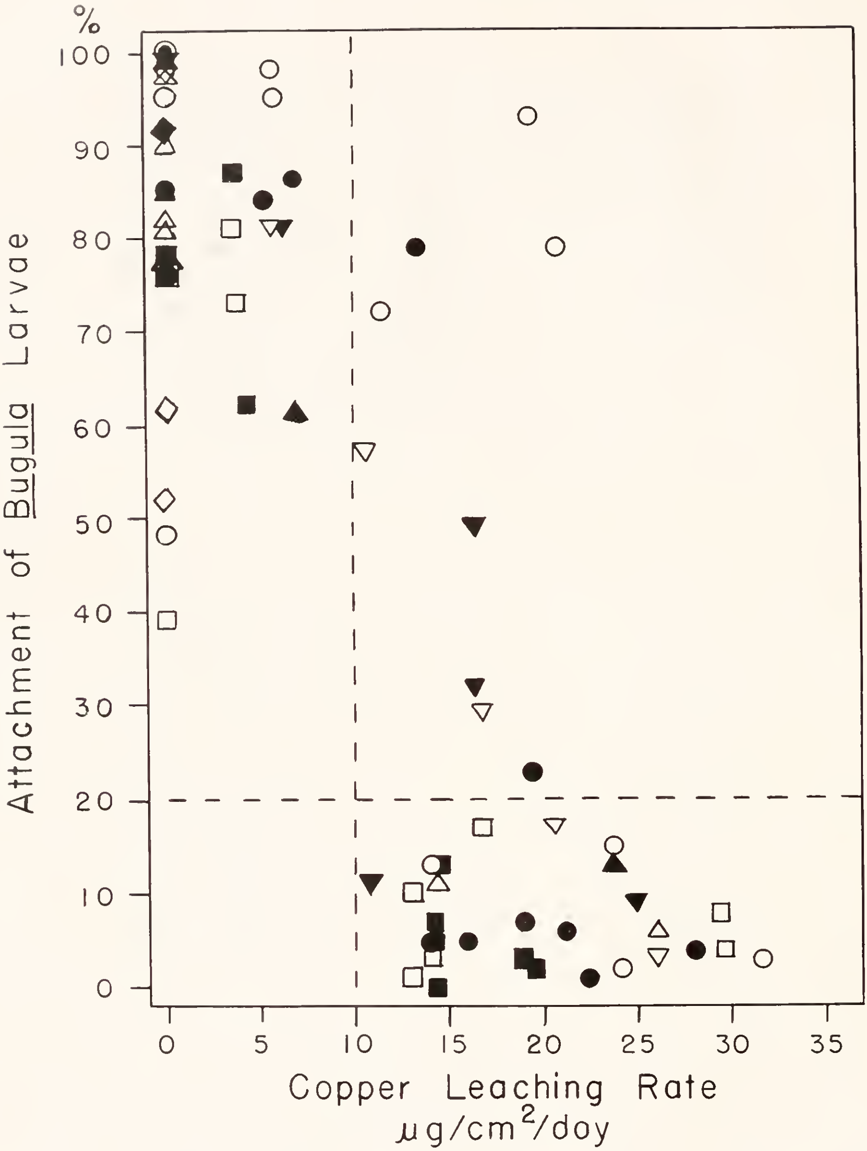

FIGURE 1. Attachment of *Bugula* larvae in relation to leaching rate and the presence or absence of slime film. Filled data points indicate results using test panels coated with slime film; clear data points indicate results with clean (defilmed) panels. Series A paints are represented by circles, Series B by squares, Series C by upright triangles, Series D by inverted triangles, and ground glass by diamond-shaped points (see Table I for description of these series).

Note that few larvae (less than 20 per cent with few exceptions) attach to panels having copper leaching rates greater than 10 micrograms per square centimeter per day (the "minimum adequate leaching rate" of Ketchum, *et al.*, 1945), regardless of the presence or absence of slime film. At leaching rates less than the minimum adequate level, most of the *Bugula* larvae attach (usually 75 per cent or more).

films that possess special repellent properties. The effect cannot be attributed to w.w. rosin alone, since the non-toxic slimes formed on the control panels in Series A were attractive to the attaching *Bugula* larvae. Substitution of ester gum for all of the rosin (as in Series C) or even for one-fourth of it (as in Series D) reverses the direction of larval attachments, i.e., the filmed surfaces are then preferred.

There is no ready explanation for the demonstrated association between the greater toxicity of slimed copper-paint surfaces and the presence of w.w. rosin as the only resinous ingredient in the paint. It cannot be explained on the basis of leaching rate differences between slimed and clean surfaces, since filmed panels coated with Series A paints have lower leaching rates than their defilmed duplicates, as is true for all series (Table II). This should favor larval attachment to filmed rather than to defilmed surfaces, but in Series A tests the *Bugula* larvae tended to avoid film-coated surfaces, even if it meant their settling on clean surfaces having higher leaching rates. Neither can the phenomenon be explained on the basis of the copper content in the slime film because slimes formed on Series B paints contain as much, if not more copper than those formed on Series A paints; yet the direction of larval preference in these two series is exactly reversed. Further biological and chemical studies are needed to clarify the effect of matrix ingredients on the toxic properties of slime films, and indeed, to learn more of the nature of these films and their components in relation to fouling.

## Acknowledgments

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## Summary and Conclusions

The presence of a slime film on non-toxic substrates facilitates, but is not prerequisite to, the attachment of *Bugula* larvae. When given a choice, they prefer to settle on slime-coated rather than slime-free non-toxic paint surfaces, but, if the former are not available, the larvae will attach equally abundantly on clean non-toxic paints. Significantly fewer of them attach, however, to clean than to filmed ground glass, and practically none to clean smooth glass.

On toxic (copper-paint) surfaces, slime films may decrease, improve, or have no apparent effect on the antifouling properties, which are determined primarily by the rate of diffusion (leaching rate) of toxic ions from the paint. Slime films produced by surfaces having high leaching rates apparently do not affect the repellent action of those surfaces, since few *Bugula* larvae are able to attach whether a slime film is present or not. Films formed on moderately or slightly toxic copper-paint surfaces usually make them somewhat less repellent to attaching *Bugula* larvae, probably by decreasing the leaching rate. Sometimes, however, slime films may improve the repellent properties of toxic paint surfaces, namely, films formed on hot-plastic copper-paints containing w.w. rosin as the sole resinous ingredient. Substitution of ester gum for all or a part of the w.w. rosin, or the use of other types of matrices reverses the effect, since *Bugula* larvae then prefer filmed to slime-free surfaces.

The demonstrated association between the composition of the paint matrix and the effect of slime films cannot be explained on the basis of leaching rate differences between filmed and non-filmed surfaces, nor on the basis of the copper content of the slime. Further studies are needed to clarify the relationship between matrix composition and the nature of the slime film.

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# NUTRITION AND SEXUALITY IN PROTANDRIC GASTROPODS OF THE GENUS CREPIDULA

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It is well known that the nutrition of the Crepidulas and other fixed or sedentary ciliary-feeding gastropods depends upon the ingestion of minute organisms and particles suspended in the water. These materials are brought to the gill by ciliary action. The water in passing through the gill is filtered through sheets of mucus secreted by the cells of the gill. Many of the suspended particles are caught in this mucus which is then carried by ciliary action through the long food groove on the dorsal surface of the body and thence to the mouth. The larger particles are carried directly to the mouth from the anterior end of the gill, as so fully described by Yonge (1938).

These ingested materials consist of dinoflagellates, diatoms, bacteria and other phytoplankton, together with various kinds of flagellates, ciliates and other protozoa, ova and spermatozoa of invertebrates, and a large proportion of detritus derived from the disintegrated cells of any unicellular or multicellular plant or animal. Particles of sand, of mollusk shells, and other inert substances are included in the stomach contents.

Young individuals have some capacity for locomotion and obtain part of their nutrition by browsing on particles similar to those already mentioned but which may be attached to the substrate. The radula with its many teeth is an efficient organ for this purpose (Coe, 1947).

The question now arises as to the necessity for this nutrition in order that the individual may realize normal sexual development. It should be remembered that all species of *Crepidula* of which the life histories are known normally experience a functional male phase when young and later change to the female phase.

It has been shown by Coe (1936, 1938, 1938a) that the age at which the male phase becomes functional and the duration of that phase varies with the species, with the individual and with the environmental conditions. In this and related genera the functional male phase may be recognized externally by the presence of a relatively large copulatory organ, the phallus, posterior to the right tentacle. This organ usually becomes of functional size only when the gonad has supplied enough spermatozoa to fill the spermatic vesicles. Its outgrowth from the body generally begins with the onset of active spermatogenesis and its retrogression in the phase of sexual transition accompanies the termination of sperm formation. These sexual phases in *Crepidula plana* have been fully and accurately described by Gould (1917).

There are many irregularities and some exceptions to this general rule, for in some individuals of *Crepidula adunca* the phallus has already begun its growth at the time of hatching and before the onset of spermatogenesis. In both *C. fornicata* and *C. plana* the male phase may be aborted experimentally and the phallus is either not formed or it is developed to only a small fraction of its functional size, presumably because of abnormal conditions. This means that the only functional phase, if any, experienced by such an individual is female (Coe, 1936). An older individual, after having passed the period of life when the male phase would normally have appeared, seems to have lost the capacity for inaugurating that phase when conditions for normal activities are restored.

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[1] Contributions from the Scripps Institution of Oceanography, New Series, No. 364.

Not only in mollusks but in all groups of animals and plants, the extent and periodicity of the sexual processes are distinctly responsive to environmental influences. Abnormal temperature, toxic substances in the water, lack of oxygen and lack of food have all been shown to influence both the time and extent of development of both male and female phases in *Crepidula.* The extent, but not necessarily the onset, of the male phase is also influenced by association with another individual in the female phase (Gould, 1917, 1917a, 1919; Coe, 1936, 1938, 1938a).

In *C. plana,* the young snail when it leaves the protection of its mother's foot is only about 1 mm. in length but normally grows to a length of 4 to 6 mm. before beginning active spermatogenesis, with the concurrent outgrowth of the phallus. The increase in size is obviously dependent upon access to the food materials already mentioned. Microscopic examinations show that the sea-water supplied at most marine laboratories, including those at Woods Hole and the Scripps Institution of Oceanography, contains far less of materials required by the young snail than the water before it enters the supply system.

The nutrients available may be sufficient to permit a relatively slow increase in size of the body but without the usually associated development of the reproductive system. The experiment recently reported by Gould (1947) appears to be a good example of such a condition, and similar results have been observed many times in past years. The failure of the young snails to realize sexual maturity in the male phase is presumably not to be construed as due to the lack of opportunity for associating with larger individuals as Gould (1917a, 1919, 1947) supposed but partly to lack of sufficient nutrition. Under similar conditions, but with a suitable food supply, the normal male phase is realized in a large proportion, but not in all, of the experimental animals.

If instead of keeping the young snails in clean dishes of running water they are placed in jars of water containing *Ulva* and the slimy algal growths from other aquaria or mud-covered stones from near low-water level on the seashore, the necessary microorganisms and detritus will become available and normal development often occurs. The snails may be kept separately in vials open at both ends if desired. Mosquito netting tied over the ends of the vial will keep the snail from wandering and the outgrowth of the phallus can be watched without disturbing the occupant. The overflow water from a well-stocked aquarium is also suitable. It is equally satisfactory to keep the open-ended vials or empty mollusk shells in a wire-screened box immersed in the sea. It is common experience that young individuals of oysters, clams, mussels, tunicates and other ciliary-feeding invertebrates cultured in the water at marine aquaria likewise usually realize sexual maturity only when additional food is supplied, although similar individuals develop normally in the open sea.

In nature, *Crepidula plana* occurs not only within large snail shells, often occupied by hermit crabs, but also on other objects, including wooden structures, stones, shells of many kinds and king crabs. In such situations the young may wander far from any other individual. Such solitary individuals are often found in the fully-developed male phase, although their instincts lead them to find another individual if possible. Others are left far behind when a hermit crab carries the mother and her companions away. It is obvious that such isolated individuals although sexually mature, must remain sterile throughout life unless young male-phase individuals find them after they have reached the female phase.

It is concluded from these observations and experiments that the development of the male phase in young individuals of *Crepidula plana* does not require that a mysterious "male-producing stimulus" should be transferred from an older individual provided that an adequate supply of suitable food is available, together with other normal environmental conditions. And this is likewise true of all the other species of the genus which have been investigated experimentally, namely: *C. adunca, fornicata, lingulata, nivea* (*nummaria*), *norrisiarum* and *onyx*. Nevertheless the masculine characteristics are usually more highly developed and are retained longer in individuals that are associated than in those that remain solitary.

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# REPLICATION OF SUBSTRATE DETAIL BY BARNACLES AND SOME OTHER MARINE ORGANISMS

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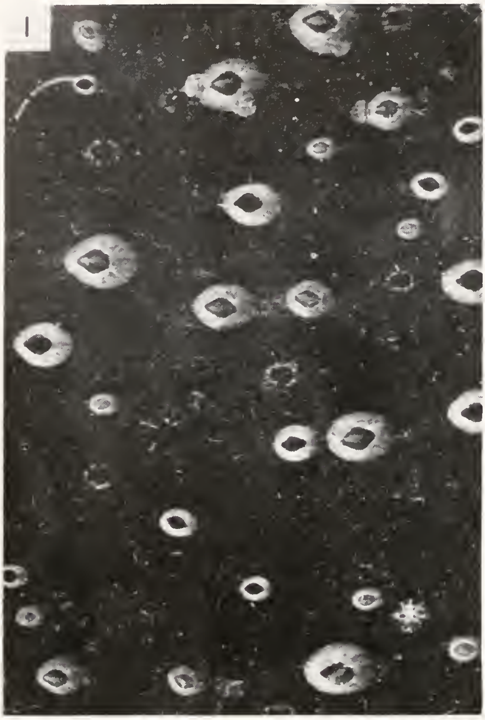
Many marine organisms exist for a short time as free swimming larvae before attaching themselves to a suitable surface and entering a new phase of their life cycle as sedentary individuals. Among these organisms are the acorn barnacles and some species of molluscs. Pomerat and Weiss (1946) found that glass plates with various surface textures to which the cypris larvae of the barnacles *Balanus amphitrite niveus* Darwin and *Balanus improvisus* Darwin attach does not affect the frequency of successful attachments. However, the writer found that the nature of the surface affects the orientation of the long and short axes of *Balanus eburneus* Gould and the topography of the calcareous compartments of *Balanus eburneus* Gould and the shells of the mollusc *Ostrea virginica* Gmel. Furthermore it is evident that the basal plate of the barnacle is subject to variations in form and topography according to the surface upon which growth takes place.

In order to study orientation phenomena and variations in form, it was necessary to use a surface of attachment affording consistent irregularities and for this purpose ordinary phonograph records were well adapted.

The records were submerged in horizontal and vertical positions several feet beneath the lowest tide level in the Mississippi Sound at Bayou La Batre, Alabama, and at the U. S. Fish and Wildlife Service laboratory on Santa Rosa Sound at Pensacola, Florida. Barnacle and oyster larvae of the species mentioned were found to attach readily and in large numbers to the black surfaces of the records. The age and size of the organisms ultimately collected depended, of course, upon the length of time the records were allowed to remain in the sea. The earliest examination of the records was made following a fourteen-day period of submergence. Many of these barnacles were assumed to be twelve to fourteen days old with regard to sedentary life, as it is possible for very large numbers of barnacle larvae to become attached during the first forty-eight hours of the submergence of a surface (Pomerat and Reiner, 1942).

The grooved surface offered by the phonograph records influenced the orientation of the barnacles to the extent that on one record 443 barnacles of a sample population of 663 had grown with their long axes parallel to the grooves in the record while the remaining 220 grew at various other degrees from parallel (Fig. 1). The 95 per cent confidence interval for this sample indicates that of the total number of barnacles attaching to the record, some 63 per cent to 71 per cent orient themselves

## Plate I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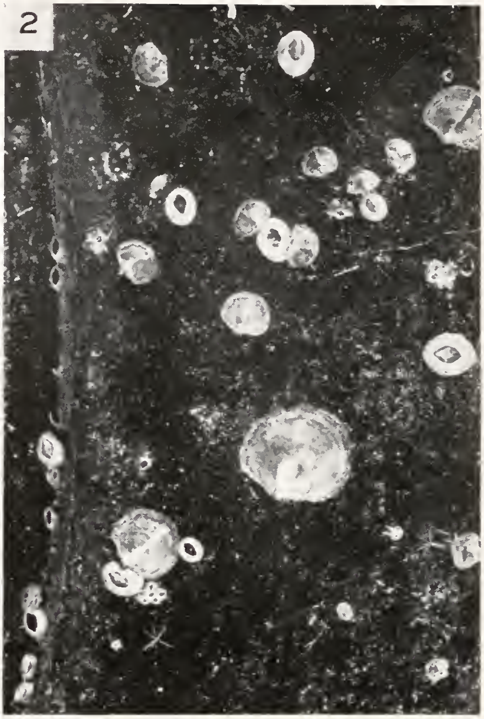
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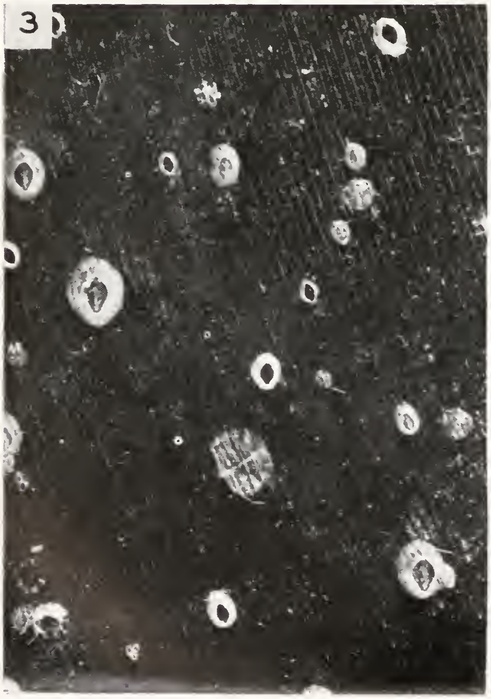
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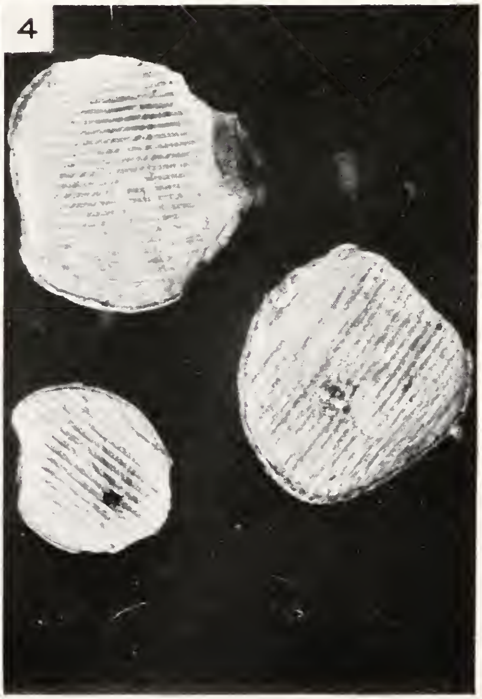

4

parallel to the grooves while not more than 37 per cent orient themselves at degrees other than parallel. The barnacles attached to the smooth surfaces of the records used as controls were oriented at random (Figs. 2 and 5). This fact tends to eliminate the possibility that geotropic or phototropic factors are greatly involved in the orientation of cypris larvae parallel with the grooves of the record. The oysters whether on the smooth or the grooved surface of a record did not show any tendency to become oriented and were attached at random (Figs. 2 and 3).

The external surfaces of the calcareous compartments of the barnacles were found to have duplicated the grooved pattern of the record while the basal plates which were in immediate contact with the record were found to have become ridged in a pattern similar to that of the record (Figs. 3, 4, 6, 7, 8). The grooves were of approximately the same width and depth and were continuous with those of the record. The grooves which appear on the compartments of the barnacle persist during subsequent growth although they were inclined to the right or left with respect to the grooves in the record with which they were continuous (Fig. 8). This shifting in space laterally occurs because of the movements of the individual compartments in the course of growth. The grooving of the record was not found to have affected the internal surfaces of the basal plate or the compartments of the barnacle. The terga and scuta were not affected in any way either externally or internally.

The ratio between length and width of *Balanus eburneus* Gould in many instances was affected by the presence of the grooves of the record and in consequence the shape of the barnacle was changed during growth. Regardless of the orientation the particular compartments and the parts of the basal plate of the barnacle that had to continue growth in a direction perpendicular to the grooves in the record were retarded in the rate of increase in length or width in the plane of attachment (Fig. 7).

This grooved and ridged condition was also found on the external and internal surfaces of both shells of the oyster, the grooved pattern of which was located on the external upper shell and the ridged pattern of which was located on the surface of the shell in contact with the record (Fig. 3). The grooves and ridges were well defined in the young oyster over the entire upper and lower shells both internally and externally, but they became somewhat obliterated after several months of age except at the extreme growing edges.

Other species of barnacles *Balanus improvisus* Darwin and *Balanus amphitrite niveus* Darwin were investigated by the use of phonograph records by Dr. C. M.

### Explanation of the Figures

A comparison of living barnacles and oysters growing on the grooved and smooth surfaces of phonograph records. All barnacles and oysters are of the species *Balanus eburneus* Gould and *Ostrea virginica* Gmel., respectively.

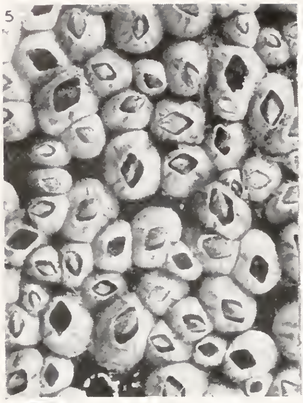
1. Orientation of the long axes of the barnacles parallel with the grooves of the phonograph record. (× 4)

2. Random orientation of barnacles and oysters on the smooth surface of the record. Note that several barnacles are oriented along the groove at the left edge of the figure. (× 4)

3. Random orientation of the oysters with the grooves of the record and showing grooves on the upper shell of the oyster (lower middle of figure) and grooves on the barnacles. (× 4)

4. Several barnacles removed from the phonograph record showing the acquired ridging on their basal plates. (× 4)

PLATE II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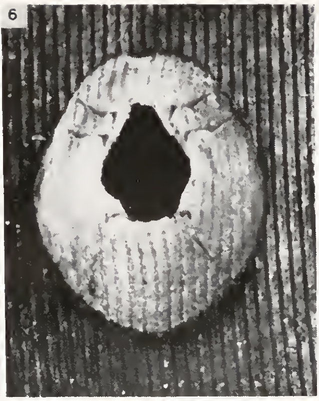
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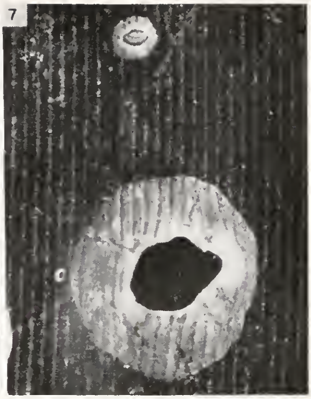
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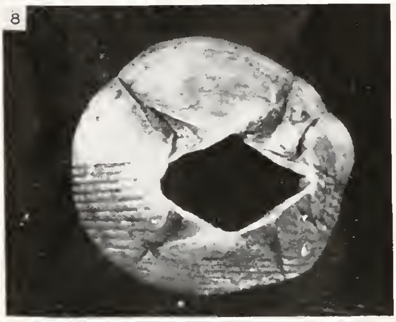
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EXPLANATION OF THE FIGURES

A further comparison of living barnacles growing on phonograph records, a fossil barnacle, a living mollusc, and a fossil brachiopod attached to various substrates.

5. Random orientation of *Balanus eburneus* Gould growing on the smooth surface of a record. (× 4)

6. Grooving on the compartments of the barnacle *Balanus eburneus* Gould which are continuous with the grooves in the record. (× 12)

7. Two barnacles (*Balanus eburneus* Gould) growing with their long axes perpendicular to the grooves of the record showing a marked departure from the normal length-width ratio and showing grooving of the compartments. (× 12)

Pomerat (personal communication). These species, however, failed to show any such striation of the compartments. This would suggest a different pattern of calcium deposition in compartment formation.

Pilsbry (1916) found that the living barnacles, *Balanus rostratus apertus* Pilsbry and *Balanus balanus* Linnaeus, when growing on *Pecten* were capable of reproducing the ribs of the scallop's shell on its own compartments.

Darwin (1854) offered an explanation for the fact that the shape of sessile barnacles depended on their position and grouping while the surface of attachment has a great effect on that of the shell. As the walls are added to at their bases, every portion has at one time been in close contact with the supporting surface and, therefore, a strongly ribbed species such as *Balanus porcatus* DaCosta might closely resemble a nearly smooth species such as *Balanus crenatus* Bruguière. However, both were found to have a peculiar appearance owing to their attachment to a species of *Pecten*. Furthermore specimens of *Balanus patellaris* Spengler have been found which were curiously pitted like the wood to which they had adhered.

Among the fossil forms which exhibit similar phenomena is the barnacle *Balanus concavus* Bronn from the Miocene found attached to a ribbed mollusc from which it had obtained a ridged surface (Fig. 9). Pilsbry (1930) found that the compartments of the fossil barnacle *Balanus shilohensis* (Pilsbry) of the Miocene were smooth except when adventitiously sculptured by growth on a ribbed shell or other rough support.

A mollusc, *Anomia simplex* d'Orb, which was found growing on a ridged *Pecten* shell, exhibited the persistence of acquired ridging and grooving on its own shell (Fig. 10).

Hall (1870) found that the surface of the valve of the brachiopod *Crania scabiosa* (Hall) [*Petrocrania scabiosa* (Hall)] was sometimes obscured by the roughness of the substance to which it is attached and caused irregular growth by which it often assumed the features of the foreign body. Figure 11 shows the resulting irregularities of an Upper Ordovician *Petrocrania scabiosa* (Hall) when growing on the shell of *Rafinesquina ponderosa* Hall. The fossil brachiopod *Crania socialis* Hall [*Petrocrania scabiosa* (Hall)] was also reported by Hall (1892) to have maintained the marks of the sutures between the segments of a crinoid to which it was attached. Among the Pelecypods attachment by cementation has been found to result in a strongly vesicular structure in the attached shell. That this phenomenon may be a more general rule is emphasized by certain attached annelidan tubes such as *Cornulites proprius* Hall. Furthermore marked vesiculation of the shell has been found among brachiopods such as *Petrocrania* and *Richthofenia*, which are attached by a considerable surface of the shell (Hall, 1892).

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8. The barnacle, *Balanus eburneus* Gould growing both on the grooved and the smooth surface of the record. Note the angle of the grooves on the compartments with respect to the surface of the record. (× 8)

9. The fossil barnacle *Balanus concavus* Bronn attached to a *Pecten* showing grooving on its own compartments. (× 1.5)

10. The living mollusc *Anomia simplex* d'Orb attached to a *Pecten* showing grooving on its own shell. (× 2.5)

11. The fossil brachiopod *Crania scabiosa* (Hall) [*Petrocrania scabiosa* (Hall)] showing the acquired ridging derived from the shell of *Rafinesquina ponderosa* Hall. (× 1.5)

Kozlowski (1929) found that the fossil brachiopod *Philhedra mimetica* Kozlowski growing on the shell of *Camarotoechia* (*Wilsonia*) *tarda* (Barrande) reproduces the ridges. In addition the fossil brachiopod *Philhedra crenistriata* (Hall) was found capable of reproducing the substrate upon its own shell.

## Discussion

The tendency for larvae of the barnacle *Balanus eburneus* Gould to become oriented with their long axes parallel with the grooved surface offered by the phonograph record was regarded as having occurred at the initial attachment when the cypris larvae were of an elliptical shape. Although the barnacles exercise slight movements in becoming firmly attached immediately following metamorphosis from the cyprid stage to the adult, the majority of them maintain their original position. It is quite possible that because of the elongate shape of the barnacle cyprid it more easily attaches itself parallel to the grooves than in other positions. The fact that oyster larvae are more or less radially symmetrical would seem to eliminate any possibility other than random chance of establishing their body axes in a particular direction when attaching to a phonograph record.

It is well known that the increase in length of the individual compartments of the barnacle is due to the secretion of building material from the lower edges of the valves (Darwin, 1854) and at the outer edges of the basal plate. This is substantiated by the fact that the grooves on the barnacle persist to the upper edges of the compartments. The secretion of the shell building material onto the grooved substrate leaves no other course for the building material than to arrange itself to conform with the pattern offered by the substrate. With subsequent secretions the building material is arranged similarly and eventually a continuous groove or ridge appears.

The oyster also became grooved and ridged while growing on phonograph records, as did an *Anomia simplex* d'Orb when attached to *Pecten* due to the fact that the mantle secreted the building material onto the available substrate and the material was obliged to follow the contours of the surface. In these two molluscs the lower shells reproduce the characteristics offered by the substrate and, therefore, the upper shells conform to the pattern conceived by the lower shells.

There are variations in the normal length-width ratio of many barnacles growing on a grooved surface. This may be attributed to the following fact. In order for the compartments and the parts of the basal plate of the barnacle growing perpendicular to the grooves in the record to attain the same linear proportions at the same rate as would its partner on a plain surface, a greater total surface area must be covered by the shell building material and in consequence a greater amount of time and material is required.

While all species of barnacles adhere closely to the substratum, not all barnacles reproduce the pattern of the substrate upon which they grow. It would seem that the species which do so may gain some selective advantage by the mechanical mimicking of a substrate pattern upon their compartments. This duplicating phenomenon is probably not merely a consequence of gaining an adequate fixation to the surface.

## SUMMARY

1. Barnacles of the species *Balanus eburneus* Gould were found to orient themselves with their long axes parallel to the grooves in the substrate, while oysters (*Ostrea virginica* Gmel.) were found to orient themselves at random.

2. The grooving in the substrate was found to affect the topography of the compartments and the basal plate of the barnacle *Balanus eburneus* Gould. The grooves are duplicated upon the compartments and the ridges upon the basal plate. Any one groove on the record causes a ridge to occur on the basal plate and a groove to appear on the compartments of the barnacle. These grooves and ridges are initiated at the early growth stages and persist irrespective of the ultimate size of the organism.

3. In a similar manner the grooved surface of the phonograph records affects the shells of the mollusc *Ostrea virginica* Gmel. both externally and internally by causing grooves and ridges to appear on the upper and lower shells.

4. The living species of barnacles, *Balanus rostratus apertus* Pilsbry, *Balanus balanus Linnaeus* (Pilsbry, 1916), *Balanus porcatus* DaCosta, *Balanus crenatus* Bruguière, *Balanus patellaris* Spengler (Darwin, 1854), and the fossil barnacles *Balanus shilohensis* (Pilsbry) (Pilsbry, 1930) and *Balanus concavus* Bronn also have their compartments affected by the substrate to which they attach.

5. The mollusc *Anomia simplex* d'Orb may also exhibit characteristics of the surface to which it attaches.

6. The fossil brachiopods *Crania scabiosa* (Hall) [*Petrocrania scabiosa* (Hall)] (Hall, 1870), *Philhedra mimetica* Kozlowski (Kozlowski, 1929), *Philhedra crenistriata* (Hall) and the annelid tube of *Cornulites proprius* Hall (Hall, 1892) have been found to assume certain features of the substrate upon which they grew.

7. Grooving and ridging occur on the barnacle *Balanus eburneus* Gould because the shell building material secreted by the organism in maintaining close contact with the substrate is obliged to follow the contours of the surface. This close association during growth eventually leads to the formation of grooving on the compartments and ridging on the basal plate.

8. A similar explanation for the fact that young of the molluscs *Ostrea virginica* Gmel. and *Anomia simplex* d'Orb show ridging and grooving phenomena is offered, with the exception that the lower shell of a mollusc acts as the pattern to which the upper shell must conform during growth. This explanation is probably adequate for similar substrate reproduction by the brachiopods.

9. Variations in the normal length-width ratio of *Balanus eburneus* Gould result from growth upon a grooved surface. This was attributed to the fact that the compartments and the parts of the basal plate which must grow perpendicular to the grooves are retarded in the rate of increase in length or width in the plane of attachment.

## ACKNOWLEDGMENT

The author gratefully acknowledges the advice which was given by a number of friends here and elsewhere. Appreciation is extended to Professor Benjamin Howell for his resolution of the problems of paleontologic taxonomy. By the kind permission of Dr. A. E. Hopkins some of the work was carried on at the U. S. Fish and Wildlife Service laboratory at Pensacola, Florida.

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# SPINDLE DEVELOPMENT AND BEHAVIOR IN THE GIANT AMOEBA[1]

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The anaphase movement of chromosomes has long been a problem, many details of which are still unsolved. The elongation of the interzonal region of the spindle as first postulated by Belar, seems to play an important part in forcing groups of daughter chromosomes apart. The mechanism by which this elongation is accomplished has not been made clear. Evidence bearing on the question was presented by Short (1945), who found 20 anaphase and telophase spindles twisted to the right in the giant amoeba, when the groups of daughter chromosomes were over 17 microns apart. None were twisted to the left. "The constancy in the direction of the twisting would result from a property inherent in the spindle apparatus; possibly an uncoiling of spindle elements as elongation proceeds." Since no previous observations of interzonal twisting, constant in direction, have been recorded, and the observations of Short are limited to 21 spindles in one amoeba, it is important to determine whether the direction of twisting is always the same in different individuals and whether this twisting is always associated with elongation. Such determination may add much to our understanding of the mechanism of elongation and of the nature of the interzonal fibers as well.

The giant amoeba is favorable material for a study of the mitotic spindle. It is easily cultured in the laboratory and multiplies rapidly. It possesses a large number of nuclei, all of which divide at essentially the same time. These dividing nuclei are not confined within a limited space. The majority of divisions allow for spindle elongation without obstruction by a nearby wall or cell membrane. The living amoeba has a somewhat characteristic appearance at different mitotic stages, making it possible to select desired stages for study. The work reported here has been confined to those stages of mitosis in which the spindle is apparent, and has been concerned primarily with the direction and constancy of interzonal twisting.

Throughout this paper the term *interzonal* will be used when referring to that part of the spindle which is seen between the separating groups of daughter chromosomes. This part may be made up of continuous fibers as well. The term chromosomal fibers has been applied to that part of the spindle extending from each group of daughter chromosomes toward the polar region. The direction of twisting of the interzonal spindle has been referred to as "right-hand" or "to the right." This is intended to mean that if one observes a *side-view* of an interzonal spindle (as seen from either end, not to be confused with a polar-view) fibers at the uppermost focal level are seen to cross from the observer's left side of the nearest chromosome plate, to the observer's right side of the more distant plate, while fibers at the lowermost focal level cross from the observer's right of the nearer plate to the observer's left of the distant plate.

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[1] The proper name of this organism has been the subject of considerable controversy for a number of years. It is variously known as *Chaos chaos*, *Pelomyxa carolinensis*, and *Amoeba carolinensis*. Recent discussions of the validity of the various names have been presented by Short (1946), Kudo (1946), and Wilson (1947). Authorities for the taxonomic names are found in these papers.

[2] This work was done at the Miller School of Biology, University of Virginia, in the session 1945–46. It fulfills in part the requirements for the degree of Master of Science. The writer wishes to express his indebtedness and sincere appreciation to Dr. Ladley Husted for helpful advice, assistance and encouragement, and to Mr. R. B. Short, who made his slides available.

Observations made during the course of this study show that "pushing" forces are involved in anaphase separation. The data obtained show that a right-hand twist of the interzonal spindle, accompanied by an apparent relaxation of the fibers as the separation of the groups of daughter chromosomes continues, consistently appears when these groups have moved 18 to 20 microns apart.

## Material and Methods

The original stock culture was obtained from the General Biological Supply House, Chicago, Illinois, in December 1945. The amoebae were grown in butter dishes half-filled with wheat culture medium, containing paramecia, rotifers, bacterial gloea, numerous ciliates and water molds. No difficulty was experienced in maintaining cultures in vigorous condition at ordinary laboratory temperatures at any time of the year.

Specimens selected from stock cultures were fixed with Carnoy's fixative, and stained with either Delafield's or Heidenhain's haematoxylin. The amoebae were mounted in diaphane direct from absolute alcohol or cleared in xylene and mounted in balsam.

Amoebae undergoing nuclear division have considerable depth at all of the mitotic stages. When they are placed on a slide they tend to become flattened if the surrounding water is partially removed. Further flattening is obtained by dropping the fixative directly on the animal from a pipette. This flattening facilitates the process of staining and destaining, as well as later study. Since, however, the possibility exists that the cytoplasmic movements involved in the flattening process might cause distortion of the spindles, few slides were made by this method. The majority of the animals studied were either dropped directly into the fixative, or were flooded with fixative before a detectable change in shape occurred.

## Development and Behavior of the Spindle

### *Mid-prophase*

Spindle fibers first make their appearance during mid-prophase. At this time the nuclear membrane is intact, and the nucleus appears as a flattened sphere, measuring 22 × 19 microns. The chromosomes, which are numerous and small, have become loosely grouped on a plate measuring 18 × 14 microns in polar-view. The chromosomal fibers are fairly distinct, lying approximately at right angles to the chromosome plate, and measuring four microns in length.

### *Late-prophase*

At a slightly later stage of prophase, first described for the giant amoeba by Kudo (1947), and similar to a stage described and pictured by Liesche (1938) for

*Amoeba proteus,* the chromosomes are well oriented on a plate measuring 13 × 11 microns, and the nuclear membrane is sharply contracted until it is in contact with the ends of the chromosomal fibers. The fibers are four to five microns long, and are definitely, although not greatly arched. The entire nucleus in edge-view appears as a slightly rounded rectangle. Kudo (1947) in his description of this stage states that "the spindle fibers undergo further development and extend to the nuclear membrane." This is not in agreement with the observations of the writer. The fibers do not appear to increase appreciably in length, but rather the nuclear membrane contracts until it is in contact with the fibers.

### *Metaphase*

The metaphase figure presents much the same picture as the late prophase stage described above, but the nuclear membrane has now disappeared, and the chromosome plate is more compact, measuring 11 × 9 microns. The arching, or curvature, and length of the chromosomal fibers show no appreciable change.

### *Anaphase-telophase*

In an unflattened amoeba containing many nuclei, the anaphase or telophase figures are found distributed throughout the animal. They are found at all focal levels with their long axes vertical, horizontal and tilted at various angles. There are significantly more side-views than polar-views, as might be expected, since the depth of the animal is less than its other dimensions, but in other respects the distribution of figures is a random one. In some regions few figures are seen, while in others many may be found in close proximity. In an amoeba 60 microns thick, and containing many food vacuoles, some anaphase or telophase figures will inevitably be partly obscured, and perhaps a few entirely hidden. The spindles of strictly polar-views were not possible to analyze, and the same was true of some part-polar-views and obscured side-views (Table I).

Many different degrees of daughter chromosome separation were studied, involving more than forty amoebae. Distances between daughter groups varied from less than one micron to sixty-five microns. The appearance of the figure changes considerably as the groups of daughter chromosomes move progressively farther apart. Early stages continue to show the arching of chromosomal fibers observed at late prophase and metaphase, but this curvature is gradually lost. The chromosomes of each daughter group become increasingly closely aggregated on the plates. As the plates decrease in diameter the chromosomal fibers become more closely packed and straight. The outside chromosomal fibers at late anaphase are inclined at a sixty degree angle to the plate. They do not change materially in length but remain four to five microns long throughout division.

Early anaphases show the interzonal fibers as straight lines from plate to plate. Later, however, when the chromosome plates have moved farther apart, there is a pronounced barreling of these fibers—the diameter of the interzonal spindle being considerably greater at its center than its diameter close to either plate.

The appearance of granules along the ends of the chromosomal fibers has been taken to be the beginning of telophase since it is along the region of these granules that the daughter nuclear membrane forms (Short, 1946). If this is used as a criterion for the beginning of telophase, then there is an overlapping of anaphase and telophase stages with respect to the length of interzonal fibers. Granules at the ends of chromosomal fibers and even developing nuclear membranes have been observed when the groups of daughter chromosomes were as little as 20 microns apart, while more often groups much farther apart show no evidence of granules or membrane. When the new membrane has completely formed, both chromosomal and interzonal fibers disappear.

The fibers of the interzonal spindles, which are barreled, at mid-anaphase lie approximately parallel to one another. This barreling of the interzonal spindle clearly indicates that its elongation is meeting with resistance. Occasionally figures are seen in which daughter groups of chromosomes have encountered obstacles to their movement. In one such instance two anaphase figures, elongating in the same plane, met end to end. The two sets of chromosomal fibers which collided were crumpled, and interzonal fibers of both figures were buckled. Similar effects were observed in cases in which one or both groups of chromosomal fibers encountered a food vacuole or the pellicle. It seems evident that the pushing forces of elongation continue even when further separation of groups of daughter chromosomes is prevented.

When interzonal spindles reach a length of 18 to 20 microns, barreling of the fibers disappears. The fibers now appear relaxed. Accompanying this apparent relaxation is a pronounced and consistent right-hand twisting of the interzonal spindle. In no amoeba studied at or beyond this stage, were the interzonal spindles either straight or twisted to the left (Table I). A very few spindles showed the right-hand twist when only 15 microns long. These were in animals in which most spindles were considerably longer. Elongation of the spindle continues and the twisting persists. The longest interzonal spindle which could be analyzed measured 45 microns, and showed a distinct right-hand twist. Many spindles of greater length were observed, the longest measuring 65 microns. These invariably had a rope-like appearance, and individual fibers could not be distinguished.

TABLE I

*Frequency of interzonal spindle twisting in two amoebae*

| Distance between plates—microns | Number of figures analyzed | | | Number twisted to right | Number twisted to left |
|---|---|---|---|---|---|
| | No. 1 | No. 2 | Total | | |
| 1–14 | 0 | 0 | 0 | 0 | 0 |
| 15–17 | 1 | 1 | 2 | 2 | 0 |
| 18–22 | 6 | 47 | 53 | 53 | 0 |
| 23–27 | 16 | 19 | 35 | 35 | 0 |
| 28–32 | 13 | 2 | 15 | 15 | 0 |
| 33–37 | 8 | 1 | 9 | 9 | 0 |
| 38–42 | 6 | 1 | 7 | 7 | 0 |
| 43–47 | 1 | 0 | 1 | 1 | 0 |
| Analyzed | 51 | 71 | 122 | 122 | 0 |
| Undetermined* | 55 | 37 | 92 | | |
| Total | 106 | 108 | 214 | | |

* Polar, semi-polar, and 27 side-views partially obscured.

Several amoebae which showed twisted spindles did not show enough figures for statistical study. Two amoebae were selected which contained a large number of figures which could be accurately analyzed. Data obtained from these two animals are presented in Table I. The two animals contained a total of 122 spindles which it was possible to anaylze. These varied in length from 15 to 45 microns. All of these spindles were twisted to the right.

## DISCUSSION

Kudo (1947) discusses the reports of Short (1945, 1946), Dawson, Kessler, and Silverstein (1935), Hinchey (1937), Liesche (1938) and Nozawa (1939) of twisting of interzonal spindle fibers at late anaphase in various amoebae. He offers the simple explanation that "the inter-plate fibers become twisted because of the violent boiling movement of the protoplasm. . . ." Unfortunately he seems to have missed the point stressed by Short that "the fact that the spindles consistently twist in one direction indicates that this twisting which accompanies spindle elongation is not a chance phenomenon brought about by cytoplasmic movement or other external factors operating on the spindle." The same reasoning that Kudo uses in criticizing a statement of Hinchey would seem to apply in this case. In commenting on Hinchey's statement that "protoplasmic streaming next moves the plates apart," he makes the well taken point that "if this is the case, the streaming must necessarily be localized and the direction of streaming must be opposite for each of the hundreds of dividing nuclei, which cannot be verified." It seems equally unreasonable to suppose that "boiling" protoplasm could produce a twisting of spindles constant in direction.

Dawson, Kessler and Silverstein (1935) report spindle twisting in *Amoeba dubia* at mid-telophase. They state that "almost invariably a twisting of the spindle occurs at this stage." These workers unfortunately do not comment on the direction of twisting, but it is interesting to note that their illustration entitled "Typical twisting of spindle cylinder" shows a right-hand twist.

There are a number of striking similarities between the appearance of the spindle at various stages of division in the giant amoeba, and those described for the uninucleate *Amoeba fluvialis* by Dobell (1914). The chromosomal fibers of both animals are short, become more closely packed as division progresses, and do not change appreciably in length throughout division. Interzonal fibers of both animals are straight at early anaphase, barrel out as anaphase progresses and appear to relax at late anaphase or early telophase. It is noteworthy in this connection that while Dobell merely states that the spindles at this stage are "often somewhat twisted," his figure illustrating the stage likewise shows a distinct right-hand twist.

Hinchey (1937), in describing anaphase movement in the giant amoeba, states that "the chromatin then separates into two plates which move along the spindle fibers toward the poles until the plates are three times their diameter apart." It seems difficult to explain how the chromosome plates can "move along the spindle fibers." The writer is in agreement with the following statement of Dobell (1914) in reference to *Amoeba fluvialis.* "It seems certain that the daughter groups of chromosomes are separated from one another by the growth of the spindle fibers lying between them—not by the chromosomes moving along the spindle fibers; for the cap-like ends of the spindle remain of the same size during the whole process, whilst the fibers between the chromosome groups become longer and longer." Such a pushing apart of the groups of daughter chromosomes by the elongation of the interzonal fibers seems the only logical conclusion to be drawn from the barreling of these fibers at mid-anaphase and their buckling when obstacles to elongation are encountered.

When the extreme elongation of the interzonal fibers is considered it seems improbable that they arise from a stretching of the chromosome pellicle or sheath, as has been suggested by various workers on other material (Schrader, 1944). The evidence here seems to be all in support of a "pushing" hypothesis. It is suggested that the development of a definite fiber between centromeres, or the poles, might bring about the separation of the metaphase chromatids and that its continued elongation might account for the further separation on the part of the daughter chromosomes.

The constancy of the direction of the twist of the interzonal spindle must be determined by the molecular structure of the fibers. The molecular organization of these fibers may well be in the form of a coil, which when uncoiled would permit a twist in only one direction, accompanied by relaxation. The hypothesis of a molecular spiral has been proposed by Darlington (1935) to explain twisting of paired chromosomes about each other as they elongate at meiotic prophase. If lateral attractions between interzonal fibers exist, then the principles involved in Darlington's hypothesis for chromosomes would apply to the twisting of the spindles.

The work of Dawson, Dobell and other investigators mentioned above suggests that twisting of the interzonal spindle might be found to be a consistent phenomenon in species other than the giant amoeba. Amoebae are considered to be especially favorable material for a study of this phenomenon. Spindles of many organisms, being confined by small cell limits, may never reach a length that allows twisting to become apparent. A greater range of organisms, in which a considerable elongation of the spindle is possible, should be investigated for evidence of the twisting here described.

## Summary

The chromosomal fibers of the giant amoeba appear during prophase, approximately at right angles to a loosely organized chromosome plate. At late prophase they are more closely grouped and slightly arched. This arching is observed during metaphase and early anaphase, the fibers becoming straight at mid-anaphase. They do not change materially in length at any stage of nuclear division. Chromosomal fibers become increasingly closely packed as the diameter of the chromosome plates decreases.

Interzonal spindles are straight at early anaphase, barrel out at mid-anaphase, and appear relaxed at late anaphase and telophase. They reach a length of 65 microns, but spindles more than 45 microns long appear rope-like and individual fibers cannot be distinguished. When groups of daughter chromosomes with their chromosomal fibers encounter an obstacle to their movement, chromosomal fibers are crumpled, and interzonal fibers buckle. This behavior, and the barreling of mid-anaphase spindles, must be interpreted as evidence that anaphase separa-

tion in this organism consists of a pushing apart of the groups of daughter chromosomes by the elongation of the interzonal spindle.

Interzonal spindles 18 or more microns long are consistently twisted to the right. Uncoiling of a spiral of constant direction within the interzonal fiber, accompanied by a lateral attraction between fibers, is offered as a possible explanation of the constancy of the direction of the twist.

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# CALCIFICATION IN MARINE MOLLUSCS *

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## Introduction

The correlation between mantle activity and the production of shells in molluscs has been of interest for a considerable time. Studies by Rawitz (1888, 1890) have shown in considerable detail the histological structure of the mantle tissues of a number of species. Biederman (1914) and Schmidt (1923) described the morphological aspects of shell formation with particular reference to the pattern and arrangement of the various components. The reports of Moynier de Villepoix (1892), Brooks (1905), Hass (1935), Bourne (1943) and others too numerous to mention have also added considerably to our understanding of the structure, composition, crystalline pattern and in some degree to the detailed mechanism involved in the calcification of the shell.

Despite a voluminous literature (see Hass, 1935) which has appeared in connection with calcification in molluscs, several phases of this problem still await further study and clarification. By means of methods to be described in the following paragraphs we have examined in detail a number of aspects of shell formation and in so doing have utilized several different methods of attack upon the problem.

## Material and Methods

The species of molluscs used in this investigation consisted of *Atrina rigida* (*Pinna*), *Pedalion alatum*, *Codokia orbicularis, Pinctata radiata*, and *Venus mercenaria*. For studies in a closed system the young spat of *Pedalion* proved to be most successful. For rapid regeneration studies in which a glass cover slip was placed in the mantle cavity (Brooks, 1905) *Pinna* proved to be efficacious. *Codokia* and *Venus* were better suited for histological and histochemical studies than other forms examined due to the relative lack of pigment in the mantle tissues.

Standard techniques and procedures were used for histological studies. Mucin was localized by Hoyer's thionin method, phosphatase, pre-formed phosphate and reticulum according to the methods of Gomori (1937, 1941), and calcium according to the method of Feigl (1943). The artificial sea water media used in our experiments was made up according to the formula of McClendon (Rogers, 1927).

In this study we were concerned with various factors relating to calcification. It was therefore necessary to choose a number of different test animals in order that different aspects of the problem could be investigated. For certain phases of the study, histological and histochemical observations were made, for others a change in the environment of the animal was initiated, in a third set of observa-

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tions calcification was induced on a glass cover slip (Brooks, 1905), while still another set of observations were obtained by means of time-lapse photography.[1]

In order to stimulate growth of the shells in several species, a portion of the shell was removed. By means of this procedure, we were able to obtain rapid regeneration and observe a number of details related to shell deposition in a relatively short time.

## OBSERVATIONS

For an understanding of the processes which will be described later it seems appropriate at this juncture to indicate briefly the salient morphological features of the mantle of the mollusc. Essentially, the mantle consists proximally of a thin sheet of tissue which is applied to the inner surface of the shell, while distally the mantle breaks up into a number of tentacle-like folds usually three in number. During the process of shell formation these folds undulate back and forth in the region of the free margin of the shell. The greater part of the mantle is covered by epithelium which varies in character from low cuboidal to the high columnar form. Mucous glands are very numerous, widely distributed and contain granules which give a positive test for calcium phosphate.

In some respects the formation of the shell is reminiscent of the early histogenesis of membrane bone. There are two distinct processes which occur: (1) the elaboration of a fibrous organic membrane, and (2) the concentration and deposition of mineral salts.

The elaboration of the organic matrix is brought about by the activity of a layer of cuboidal cells occupying the outer surface of the middle fold of the mantle (Figs. 1 and 3). The secretion elaborated by these cells is known as conchin or periostrachum. This substance, first deposited as a thin sheet, comes to be arranged in prisms and striae in later development (Fig. 6) and according to our own and other observations (Hass, 1935) is a protein and contains one or more reducing sugars. When first elaborated it takes a positive reticulum stain, later it undergoes certain modifications in staining reactions; the prismatic portion is acidophilic, the inner portion exhibits basophilic properties.

The various steps in the process of calcification of the shell were studied advantageously by means of the "cover-glass" technique. As shown in Figures 7 and 8, the first observable indication of mineralization consists of a deposit of numerous minute granules which appear to be embedded in the surface of the conchin layer. According to our observations these granules consist of calcium phosphate, and preliminary tests[2] reveal the presence of both inorganic and organic forms.

If one recalls that the mucous tissue is extremely abundant and widely distributed in the mantle tissues and that the mucous is secreted at the surfaces of the epithelial cells (Fig. 2), it becomes readily understandable that when the tentacles of the mantle undulate back and forth on the newly formed conchin layer, the granules will naturally be deposited on its surface and adhere thereto.

Once the granules are deposited in the conchin layer, they begin to undergo a typical crystal growth such as is shown in Figures 9 and 10. Continued growth

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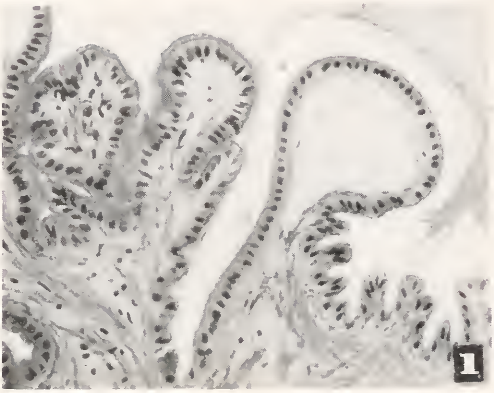
[1] Courtesy Mr. Edward Baylor, Princeton University.

[2] Tests made by Miss Lowell Lowell.

PLATE I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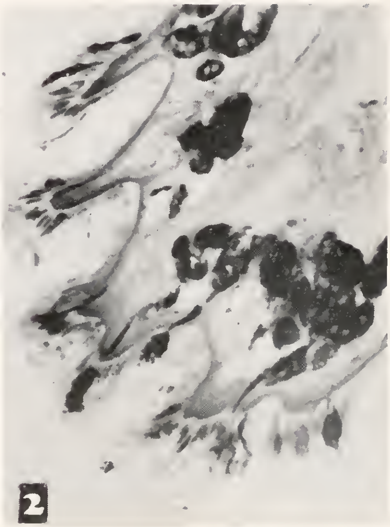
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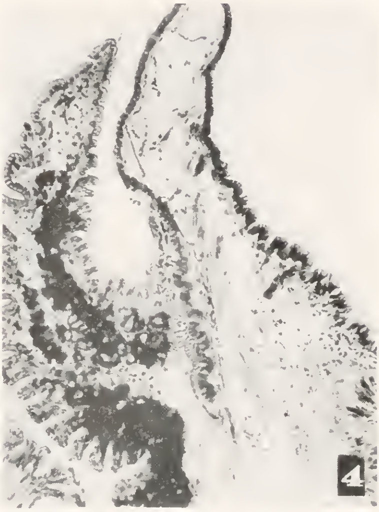
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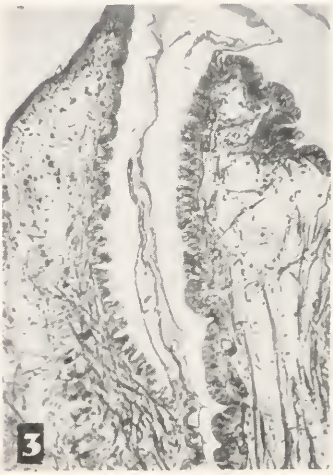
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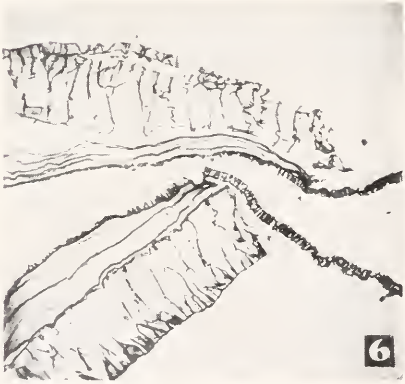
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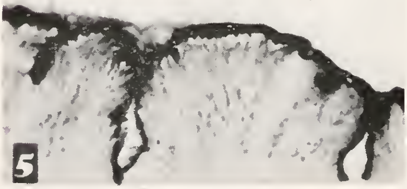
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eventually results in an arrangement whereby the crystals (in *Pinna*) come to be enclosed in a thin layer of organic matrix and assume a polyhedral shape (Fig. 11). These crystals are composed of calcite (Biederman, 1914; Hass, 1935).

One interesting cytochemical feature which was observed in this connection was the identification of a strip of epithelium covering the surface of the inner middle fold which contains heavy concentrations of the enzyme alkaline phosphatase (Fig. 5).

## Effect of Modifying Sea Water

Although lack of time did not permit a detailed study of the effect on calcification due to changes in the constituents of sea water, it was nevertheless possible to record a few pertinent observations in regard to this matter.

Using McClendon's formula for artificial sea water, young specimens of normal Pedalion were placed in this media; it was observed that they were alive and in good condition after a period of several days. Following this a number of specimens which had the free edge of the shell cut back as far as the mantle tissues were placed in this same media. Figure 12 is a photograph showing normal shell growth (regeneration) in a typical specimen.

Having shown that apparently normal calcification takes place under the conditions of the experiment, calcium was withdrawn in varying amounts from the artificial sea water and the resultant effects were observed in several specimens. The results of the experiments in which calcium was partially removed from sea water are shown in Figures 14, 15. Figure 12 shows a normal regenerating shell grown in sea water with the usual amount of calcium present. Figure 14 shows a specimen in which 50 per cent of the calcium was withdrawn, while Figure 15 illustrates a specimen grown in sea water containing only one-eighth of the normal calcium content. This latter specimen is completely devoid of calcium in the newly formed shell. Interestingly enough, however, the organic matrix was deposited in an apparently normal manner.

A few experiments were also made in which the magnesium content of the sea water was modified. Reference to Figure 13 shows that a similar condition may obtain when this mineral is withdrawn.

## Discussion

In our studies concerning the calcification of the shell in several marine molluscs we have reviewed the essential histological structure of the mantle tissues and in so doing find that we are in all important respects in agreement with the reports of

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Figure 1. Longitudinal section of mantle of *Codokia,* hematoxylin and eosin showing formation of conchin.

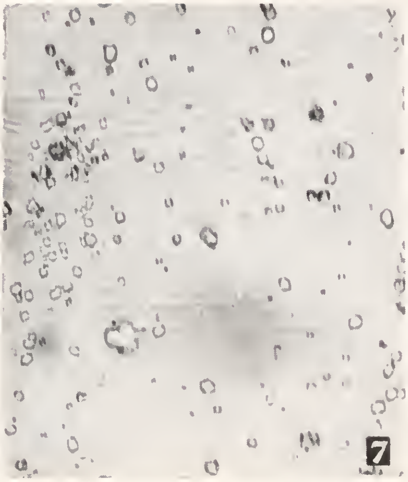
Figure 2. Section of mantle of *Codokia* stained with thionin to show secretion of mucous.

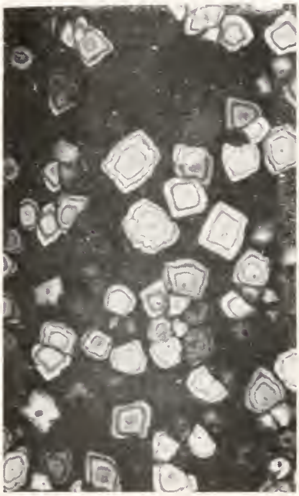
Figure 3. Section of mantle of *Venus,* hematoxylin and eosin. General topography of folds and elaboration of conchin.

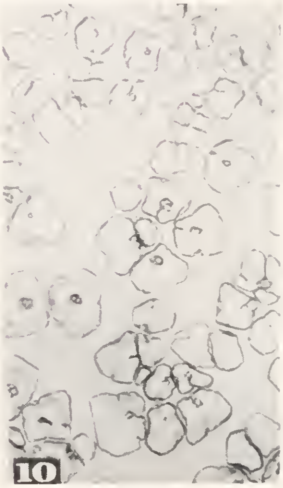
Figure 4. Mantle of *Venus* to show distribution of mucous. (Thionin)

Figure 5. Mantle of *Venus* showing distribution of alkaline phosphatase.

Figure 6. Decalcified section of shell of *Pedalion* showing arrangement of organic part of shell. Strand of tissue on right is regenerating tissue.







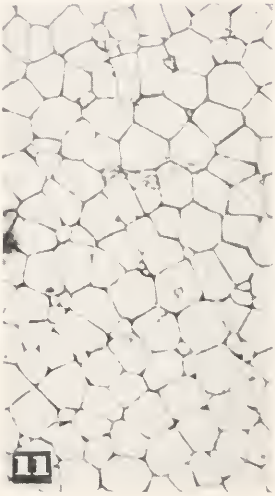


PLATE II

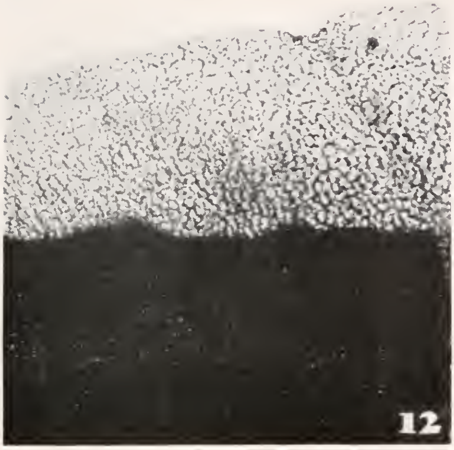
FIGURE 7. First formed elements of shell of *Pinna* deposited upon a cover slip. Note granules and fibrous matrix. Photo by transmitted light.

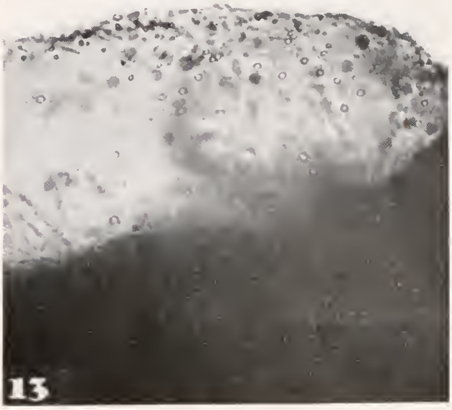
FIGURE 8. Similar preparation photographed by means of oblique illumination.

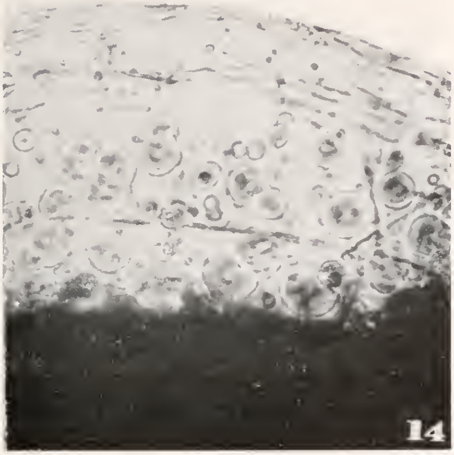
FIGURE 9. Crystal growth on cover slip preparation (*Pinna*). Photographed through crossed Nicols prisms.

FIGURE 10. Similar preparation photographed by means of transmitted light.

FIGURE 11. Mature shell (*Pinna*) surface view.







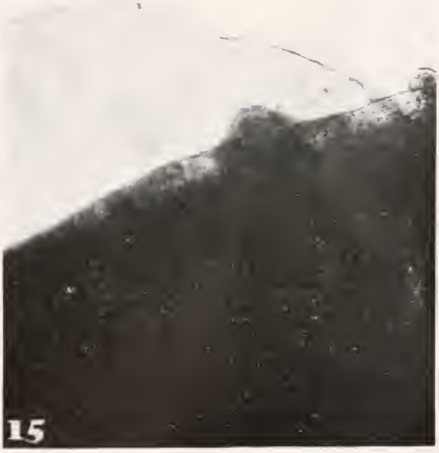


PLATE III

FIGURE 12. Normal regeneration of shell of young *Pedalion* in artificial sea-water.
FIGURE 13. Regeneration of *Pedalion* shell in which Mg was reduced.
FIGURE 14. Regenerating shell in 50 per cent normal Ca content.
FIGURE 15. Regenerating shell in one-eighth normal Ca content.

the earlier investigations (Rawitz, 1888, 1890). The observations of Biederman (1914), Schmidt (1923) and others in reference to the morphological aspects of shell formation have also been confirmed in part by our own observations.

In regard to the more minute details which occur during the process of calcification, Biederman (1914) refers to several pertinent observations and reports that while mantle tissue contains calcium phosphate in abundance, the calcified shell is made up of calcium carbonate and is completely lacking in phosphate. A similar statement appears in Plate (1922). Biederman further poses the interesting question concerning the mechanism of the transfer of phosphate to carbonate. Upon this point Moynier de Villepoix (1892) made some conjectures which seem to us at this time to have been extremely discerning: he states that it might be permissible to suppose that after the deposition of the granules in the conchin, that they might serve as the center of attraction of the elements and that the crystal growth of the calcium carbonate be determined in some manner thereby.

In regard to the presence of phosphatase on the surface epithelium of the mantle tissue, this condition was reported by Bourne (1943). Although this author's description is in many respects in agreement with our own observations, we are not certain that his photograph in fact indicates the presence of phosphatase, for our observations reveal that phosphatase is present on the surface of the cells which come in contact with the matrix and the granules, and not deeply embedded in the cytoplasm as indicated by Bourne.

In spite of the fact that many details have been omitted in this description of the process of calcification in the mollusc shell, it seems nevertheless feasible to suggest, if only in outline form, the essential steps which are involved:

An organic matrix is elaborated by epithelial cells located on the surface of the middle mantle fold. This substance gives a positive test for protein and reducing sugars. It also undergoes certain changes in arrangement and tinctorial properties as it matures.

The first observable anlage of the mineral part of the shell arises in the mucous glands, it is composed of calcium phosphate and comes to be deposited upon the matrix (conchin) due to the surface contact of the folds which pass over this tissue when the shell is being formed. The final calcified product is composed of calcium carbonate (calcite).

How this calcium phosphate is converted to the final calcified product, calcium carbonate, can be only conjectured at this time.[3] It does not seem likely that the conversion is a direct reaction between calcium phosphate and the carbonate of sea water for this process would require exceedingly high concentrations of carbonate.

Greenwald (1938) has emphasized the role played by other ions, particularly organic ones, in increasing the solubility of calcium phosphate. Such ions presumably act by binding calcium in some manner, possibly in an un-ionized moiety of the molecule. It is therefore proposed that the calcium phosphate may be dissolved by the participation of some organic ion. Phosphatase may aid this process by transferring phosphate to some substrate, thus removing the phosphate ions which otherwise would tend to slow the formation of the calcium complex. In a further phase of this process, calcium ions may be made available by the alteration of the organic ion possibly through an oxidation-reduction reaction. If high local concentrations of calcium ions were thus made available, the carbonate of the medium would slowly precipitate calcium carbonate resulting in the crystalline architecture we have noted in our investigations.

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[3] Freeman and Wilber (1948) have demonstrated the presence of carbonic anhydrase in mantle tissue and body fluids of some pelecypods and gastropods. However, these authors carefully point out that while it may have importance in shell formation in some species, negligible activity in others indicates that shell may be deposited in its absence.

## Summary

1. Various aspects of the process of calcification have been studied in several marine molluscs.
2. Calcification of the shell is brought about by the formation of an organic matrix upon which minute granules of calcium phosphate are deposited.
3. In the presence of, and in contact with the mantle epithelium, crystal growth occurs in sea water.
4. The enzyme alkaline phosphatase appears to be concerned with calcification in these forms.
5. Modification of certain constituents of sea water results in the production of a shell partially or completely lacking in mineral content.

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# REGENERATION IN PEROPHORA VIRIDIS[1]

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The potency of the Ascidians to regenerate and their susceptibility to environmental agents have long been known. Driesch (1902) found that small isolated portions of Clavellina stolon could form complete individuals. Huxley (1921) studied dedifferentiation in Perophora and later (1926) dedifferentiation in Clavellina. Berrill and Cohen (1936) studied the regeneration of isolated pieces of Clavellina stolon with respect to the polarity of the parent zooid.

Perophora, a colonial Ascidian, because of its relative structural simplicity and the uniformity of its growth pattern, seemed to offer excellent material for regeneration experiments, especially with respect to the polarity relationships of isolated portions to the colony as a whole.

The experiments to be described were carried out with the following objectives: (a) To investigate the regeneration of isolated stolons; (b) to study the regeneration of stolon tips; (c) to test the regeneration of stolonic material when expressed from the tunic; (d) to determine the effect of ligatures on isolated stolons; (e) to study the regeneration of stolon-zooid systems.

The author wishes to acknowledge the helpful guidance of Dr. Lester G. Barth during the course of this investigation.

## Material and Methods

The experiments were performed chiefly upon freshly collected Perophora colonies. Satisfactory stock material was also cultured by suspending "grape-like" clusters of colony by string in a tank of running sea water. In three or four days new stolon outgrowths, which could be handled conveniently, appeared. The experimental animals were kept in separate syracuse dishes which were immersed in a large glass aquarium through which filtered sea water constantly flowed.

A Perophora colony consists of a system of zooids appearing at fairly regular intervals from a branched stolon (Fig. 1, A). The zooids show a size seriation, becoming progressively smaller toward the free end of the colony. This seriation is due to the mode of origin of the zooid, for as the stolon grows in length buds appear near the free end and develop into mature zooids as the colony grows. Thus, the size seriation is an index of the age of the zooids, the largest zooids always being the oldest. The free growing end of the stolon in the experiments to be described will be called the distal end, as opposed to the attached end which is called proximal.

As described by Deviney (1934), the stolon of Perophora is cylindrical, its wall being composed essentially of three layers. It consists of an outer tunic with scattered cells plus intercellular substance, a middle epithelio-ectodermal layer which is quite thin, and an inner discontinuous thin layer of mesenchyme. The mesenchymal layer lines the stolonic cavity through which blood flows; this cavity is divided vertically by a single-layered septum. The septum disappears near the distal end of the stolon, allowing continuity of blood flow between the right and left halves of the cavity.

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The operations and observations were made under a binocular microscope. All cuts were made by means of a pair of iridectomy scissors. Photographs were taken with an Argus camera, attached to a compound microscope and drawings were made from these photographs.

## Results and Discussion

### *A. Regeneration of isolated stolons*

In these experiments the portion of stolon lying between two zooids was excised by two cuts and observed for regeneration (Fig. 1, B). The excised stolons varied in length from 2.5–6.0 millimeters. Either distal or proximal ends of the excised stolon were stained with nile blue sulphate suspended in agar, for purposes of orientation. The results are listed in Table I.

Table I

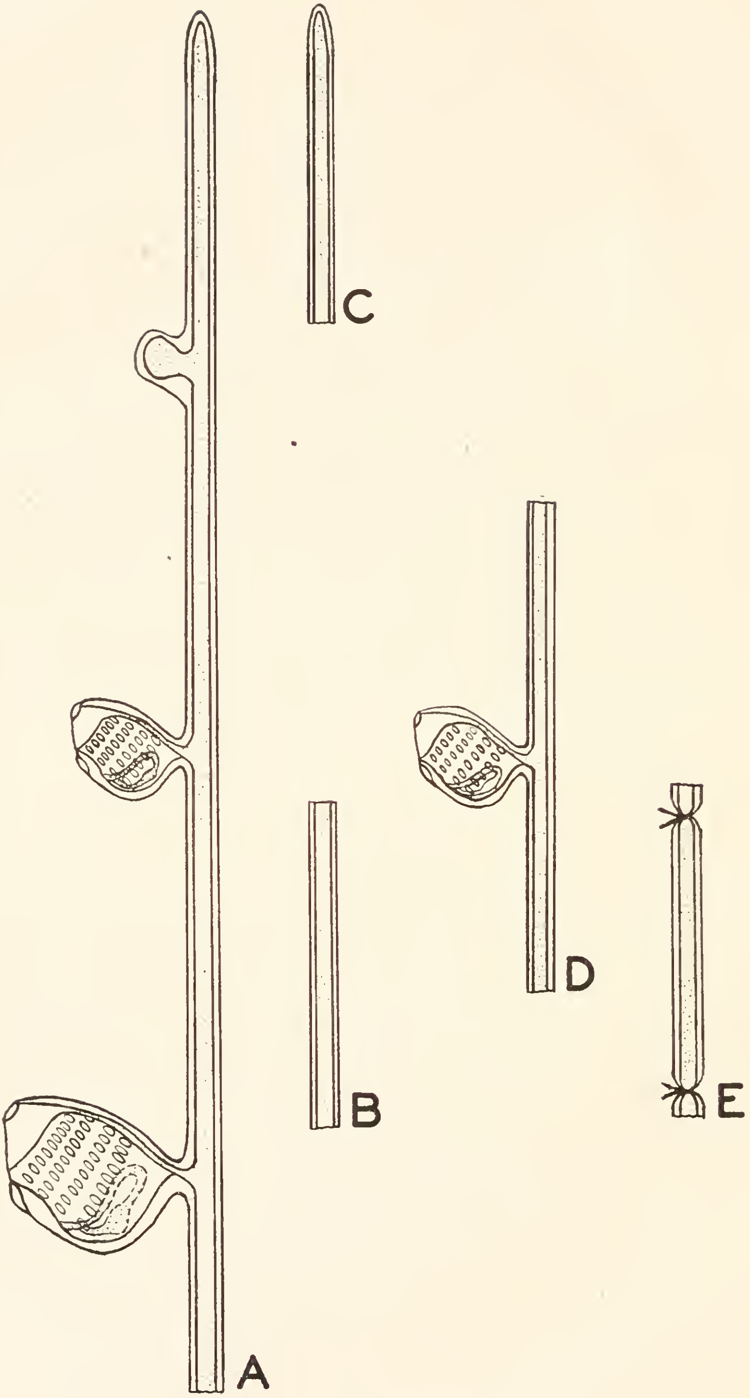
*Location of regenerants in excised stolons of Perphora*

| Location of regenerated zooid | No. of specimens regenerated |
|---|---|
| Distal | 18 |
| Proximal | 11 |
| Distal and proximal | 9 |
| On original stolon | 4 |

The regenerated buds usually appeared in about two days, and reached an approximate length of three hundred and fifty micra by the end of three days. It can be seen from Table I that bud regeneration may occur at the distal or the proximal end of the stolon, or at both the distal and the proximal ends. These buds appeared either precisely at the cut end, or on new stolon outgrowths at the cut end (Pl. I, 1–4). Bud regeneration at some point along the length of the original stolon occurred in only four cases.

These experiments show, therefore, that there is a definite tendency for regeneration to occur at the distal or proximal ends of the excised stolon. Further, there appears to be no definite polarity as regards distal or proximal regeneration. In this connection, it may be noted that the nile blue sulphate did not have an inhibitory effect on regeneration, for regeneration occurred at stained ends of the stolons.

The cases in which bud regeneration occurred at both the distal and the proximal ends of the stolon are of special interest. The two buds in most cases showed a size difference, the larger bud appearing earlier than the smaller bud. This may mean that there is a competition for metabolite between buds when more than


C
D
B
E
A

one is involved in regeneration. The smaller bud is less successful in this competition and develops more slowly. In one case, the smaller bud was eventually resorbed at the expense of the larger bud. That a competition between two regenerating regions exists is further evidenced by the fact that when bud formation occurred at only one end, there was frequently a preliminary stolon growth at the other end of the excised stolon. This stolon growth was eventually resorbed and there was a general drawing of the original stolon materials towards the developing bud (Pl. I, 2, 4).

### *B. Regeneration of stolon tips*

The portions of stolon used in this experiment consisted of distal pieces. The stolon was isolated by a cut made just distal to the first visible bud (Fig. 1, C). Of eleven such stolon pieces, varying in length from 1.7 to 4.8 millimeters, all formed buds in one to two days. These buds appeared in approximately the same position on the stolon as do new buds in normal colony formation (Pl. I, 5). No buds were observed at the proximal end of the isolated stolon, though in five cases small stolon outgrowths did appear. In two of these five cases the proximal growth was later resorbed at the expense of distal growth.

There is thus a difference in the mode of regeneration of isolated stolon tips as compared with that of stolon pieces isolated from between two zooids. In the former, the growing distal tip is probably more active in development at the time of the cut; after the cut is made, this lead is maintained. In the latter case, both ends are cut and their development may proceed on a more nearly equal basis as regards competition.

### *C. Regeneration of stolonic material expressed from the tunic*

Wilson (1923) attempted to test the regenerative power of mesenchyme cells which he pressed out of the stolon. He obtained clumps of cells which stuck together, but which died within a day. Since, as shown by Deviney (1934), in the budding of *Perophora viridis,* the unspecialized cells of the septum and of the blood stream are the formative elements, it seemed likely that with more favorable conditions the stolonic material might be kept alive, and its ability to regenerate tested.

The technique employed was as follows: The portion of stolon lying between two zooids (Fig. 1, B) was removed from the colony. Holding the tunic at one end with jeweler's forceps, a glass needle was passed gently over the tunic and the inner stolonic material was expressed from the opposite end. It was found that the inner stolonic material could be removed as a unit in this manner. The expressed stolonic material was placed in syracuse dishes and maintained in running, filtered sea water.

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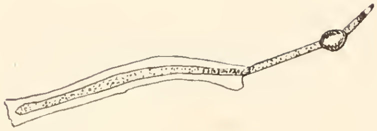
EXPLANATION OF FIGURE

Diagram to show the types of isolations which were made from the Perophora colony.

A. Perophora colony.
B. Stolon isolation.
C. Stolon tip isolation.
D. Stolon-zooid system.
E. Stolon ligatured at both ends.

## Plate I



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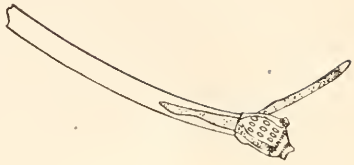

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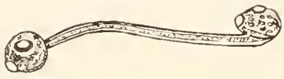

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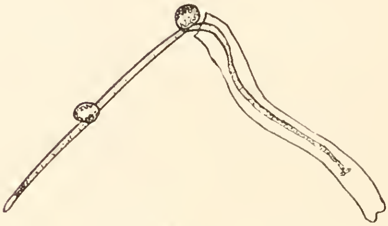

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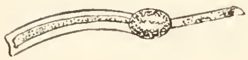

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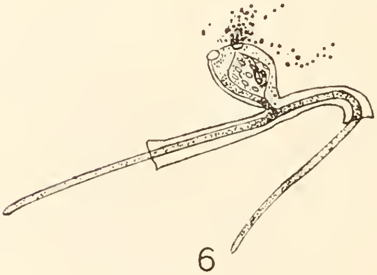

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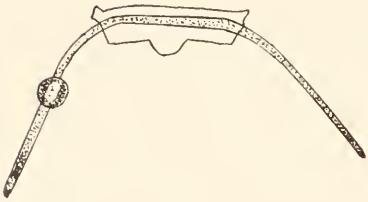

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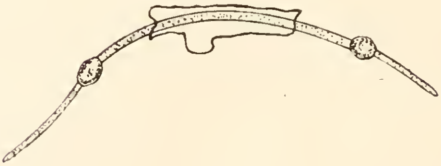

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Of a total of nineteen such cases, nine showed stolon growth and bud formation after three days, while ten showed stolon growth but no bud formation. A new tunic was observed around the stolonic materials at this time. The point of origin of new buds showed marked variation.

Since the expressed material contained the unspecialized cells of the septum and of the blood stream, it is not surprising that under favorable environmental conditions the material could survive and form buds. Wilson (1923) assumed that the material expressed from the tunic consisted of mesenchyme cells. However, Deviney (1934) has shown, as cited earlier in this paper, that there is a thin epithelio-ectodermal layer surrounding the stolonic cavity. It is possible that cells of the epithelio-ectodermal layer were included with the expressed material in these experiments, so that it may not be concluded that a particular cell type was solely responsible for bud formation. However, it is suggested that, by this method, particular cell types may be isolated, and their ability to regenerate studied.

### D. *Ligature of isolated stolons*

The thread ligature as a means of isolation was first employed by Morgan (1902), and later by Peebles (1931) and Barth (1938 a), on Tubularia. In Tubularia the perisarc remains intact after the ligature, but the coenosarc is severed, thus isolating the stem on each side of the ligature. The thread ligature acts in a similar way in Perophora. The tougher tunic is not cut, but the inner stolonic tube is effectually severed by the ligature.

Twenty-five stolon pieces, each of which was isolated from between two zooids, were ligatured at each end by means of fine cotton threads (Fig. 1, E). Twenty-two specimens consisting of similarly isolated stolon pieces, but without any ligatures, were used as controls. The results of the experiment are summarized in Table II. The ligature hindered stolon growth and bud formation, while typical stolon growth and bud formation occurred in the controls. It may be that in the one instance that regeneration occurred in the ligatured stolon, it was because the ligature was so tight that it cut through the tunic.

Barth (1938 b) has shown that the availability of oxygen is an important factor in controlling the rate of regeneration. It is suggested that in Perophora

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EXPLANATION OF PLATE I

FIGURES 1–8 (× 12)

1–4. Types of regenerants obtained after isolation of stolons removed from between two zooids. 1. Bud formation on a stolon outgrowth from the distal end of the stolon, four days after isolation. 2. Zooid formation at the distal end of a stolon, six days after isolation. 3. Bud formation at both ends of a stolon, after five days. 4. Stolon growth and bud formation at the proximal end of an isolated stolon, after five days.

5. Bud formation on an isolated stolon tip, after two days.

6–8. Types of growth and regeneration obtained after isolation of stolon-zooid systems. 6. Growth of the zooid and stolon growth at both ends of the stolon, after 6 days. 7. Resorption of the zooid, stolon growth at both ends of the stolon, and bud formation on the proximal stolonic outgrowth, after 6 days. 8. Resorption of the zooid, stolon growth at both ends of the stolon, and bud formation on both stolonic outgrowths, after 6 days.

TABLE II

*The effect of thread ligature on stolon and bud regeneration*

| | Time in days | No. of specimens | Bud regeneration with stolon growth | No bud regeneration | |
|---|---|---|---|---|---|
| | | | | Stolon growth | No stolon growth |
| Ligatured | 5 | 25 | 1 | 3 | 21 |
| Control | 5 | 22 | 11 | 4 | 7 |

there is a lowering of the availability of oxygen at the cut ends brought about by the thread ligatures, thus inhibiting regeneration.

### *E. Regeneration of stolon-zooid systems*

By isolating a normal zooid along with a short length of stolon from the Perophora colony, Huxley (1921; 1934) demonstrated a differential susceptibility of the stolon-zooid system. When the stolon-zooid system is starved under normal conditions, the stolon is resorbed by the zooid. When the stolon-zooid system is placed in dilute toxic solutions, the zooid is resorbed by the stolon. This may be interpreted as meaning that under normal circumstances there is a competition between zooid and stolon, the zooid being dominant because of a higher metabolic rate. Under moderately unfavorable conditions, the metabolically more active zooid is more severely effected, and the stolon assumes the dominant role. Thus, there is a delicate equilibrium between zooid and stolon. That region which is more active grows at the expense of the other region.

Since running sea water seems to offer the most favorable environment for survival of Perophora material, it was thought advisable to isolate stolon-zooid systems (Fig. 1, D) and place them in running filtered sea water. Presumably, under these conditions, food is available; and it was felt that further information might be obtained on the competitive relations of stolon to zooid. Each specimen was kept in a separate syracuse dish and immersed in the container through which the sea water flowed.

For the thirty-four stolon-zooid systems isolated, the results fell into three classes:

(a) The original zooid was maintained, and actually increased in size in many cases. In addition, there was stolon growth at either or both cut ends, and buds formed on these outgrowths. This occurred in thirteen cases (Pl. I, 6).

(b) The original zooid was resorbed, there was stolon growth at either or both ends, and bud formation occurred on these outgrowths. There were twenty such cases (Pl. I, 7, 8).

(c) The zooid grew at the expense of the stolon, the latter being resorbed. This occurred in only one of the thirty-four cases.

From these experiments it would appear that under the most favorable conditions, i.e. running sea water plus healthy stolon and zooid, the entire stolon-zooid system may grow. The stolon grows and may form buds, and the original zooid may maintain itself and even increase in volume. Evidence of feeding in healthy zooids under these environmental conditions has been noted (Pl. I, 6). Rather than assume a competition between stolon and zooid, from the experiment as run in this fashion, it would seem more plausible to assume that a competition occurs between three growing regions, namely the zooid and the two cut ends of the stolon. Under the most favorable conditions, when all three regions are developing equally rapidly, all continue to grow. When one gets ahead in development, it may develop at the expense of one or both of the others.

That there is this competition is demonstrated in the cases where stolon growth occurred at only one end, for in eight of these cases, it first occurred at both ends, but was later followed by stolon resorption at one end, and further stolon growth at the other end. Of the three growth zones in the isolated stolon-zooid system, the zooid appears to be most sensitive, having been resorbed in twenty of the thirty-four cases.

This explanation may be considered, then, as an extension of Huxley's ideas on equilibrium and competition, rather than as a negation of his fundamental assumptions.

## General Discussion

The results of these experiments are interesting in view of those obtained with Tubularia. Both Tubularia (Goldin and Barth, 1941) and Perophora have relatively unspecialized cells which have the ability, under the proper environmental conditions, to reconstitute the whole individual. In the normal colony formation of Perophora, buds do not normally form on the stolon lying between two zooids. In Tubularia, similarly, a hydranth generally does not form between the distal hydranth and the proximal end of the stem. A cut in either Perophora or Tubularia provides an environmental stimulus which initiates the process of regeneration at the point of the injury. Barth (1938 b) has suggested "that the stimulus for regeneration is the exposure of tissue to high oxygen." Goldin (1942 a; 1942 b) has shown that both oxygen and hydrogen ion concentration influence the rate of regeneration of Tubularia. Increased oxygen stimulates regeneration, while increased hydrogen ion concentration has an inhibitory effect on regeneration. That in Perophora, too, similar mechanisms are involved, is supported by the following evidences: In the isolation of stolons excised from between two zooids, and of stolon-zooid systems, regeneration occurs at the cut ends or on stolonic outgrowths at the cut ends. In Perophora regeneration is hindered when both ends of the stolon are ligatured, this inhibition probably being correlated with the lowered availability of oxygen and increased concentration of acid metabolites brought about by the thread ligature. Finally, when stolonic material is expressed from the tunic, providing ready diffusion of oxygen and metabolites, regeneration may occur at any point along the axis of the expressed material.

It has been shown in Tubularia that there is a definite polarity present in the stem. After isolation, distal levels exercise dominance over more proximal levels and the distal end regenerates more rapidly. In Perophora, stolons isolated from between two zooids show no evidence of original polarity relationships in regeneration. Regeneration may occur at either end or both ends, with equal frequency. In Perophora, proximal regeneration unaccompanied by distal regeneration represents a reversal of polarity, and is in marked contrast to the situation in Tubularia.

Despite this failure to maintain the original distal-proximal polarity, however, once regeneration has begun, a new competitive physiological axis is set up without any relation to the original distal-proximal orientation. This competition has been manifested in the following way: In isolated stolons regenerating new stolonic material at both ends, one end may often cause resorption of the stolonic growth at the other end. A similar situation may occur when buds form at both ends of the isolated stolon, the larger bud eventually causing resorption of the smaller bud. In isolated stolon-zooid systems there exist three active regions, namely, two cut ends, and a zooid. More rapid growth at any one of these may progress at the expense of other regions. It is interesting to note that under optimum conditions, competition is eliminated and growth occurs at all of these regions.

That factors other than oxygen availability and diffusion of metabolites are involved in the regeneration of Perophora stolons is demonstrated by experiments in which distal stolonic tips are isolated. In these stolon tips there is probably greater oxygen availability and diffusion at the cut proximal end. Despite this fact, regeneration and growth occurs at the distal end.

It is suggested that, as with Tubularia, Perophora offers a good biological system for studying, further, problems of regeneration, polarity, and differentiation.

## SUMMARY

1. Stolons of Perophora, isolated from between two zooids, regenerate at the cut ends but do not necessarily maintain their original distal-proximal polarity relationships.

2. Stolon tips, after isolation, maintain their distal-proximal polarity, the distal end displaying dominance.

3. The inner stolonic material, when expressed from the tunic, may grow and regenerate buds at any point along its axis.

4. Regeneration is hindered at ligatured ends of stolons.

5. When stolon-zooid systems are isolated and maintained under optimum conditions, the entire stolon-zooid system may grow.

6. The theoretical implications of the results of these experiments are discussed.

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# HISTOLOGY OF THE CORPORA ALLATA OF MELANOPLUS DIFFERENTIALIS (ORTHOPTERA: SALTATORIA)

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The corpora allata of insects are known to secrete a hormone called "juvenile" or "inhibitory" hormone which is responsible for maintaining the insect tissues in a juvenile condition and therefore retards metamorphosis (Wigglesworth, 1934, 1936, 1940; reviews by Hanström, 1939; Scharrer, 1941, 1948; Bodenstein, 1942; Piepho, 1943; Joly, 1945a; Mendes, 1947). They also play a role in egg production and in the development or function of male and female accessory glands (Wigglesworth, 1936; see reviews). Whether the different physiological effects of the secretion of the corpora allata are brought about by one or more than one hormone is still a controversial matter. The grasshopper *Melanoplus differentialis* has been the subject of experimental study by Pfeiffer (1939, 1945, 1945a) who has shown that hormone from the corpora allata retards metamorphosis and in adult females controls certain metabolic phenomena, the production of oviducal secretion and the deposition of yolk. It was therefore chosen for study of the histology of the corpora allata at different periods of the life history in an attempt to correlate the histological aspects with the experimental data.

## Material and Methods

*Melanoplus differentialis* passes through six nymphal stages before metamorphosis. Histological preparations were made of the corpora allata of male and female nymphs which were killed at daily intervals during the fifth and sixth stages to compare the changes in these glands during a stage that precedes the production of nymphal characters at molting with those occurring prior to metamorphosis, and to determine whether there is any sexual dimorphism in the nymphal glands. Corpora allata from adult males and females killed at significant ages were prepared for comparison of these glands in the two sexes, for comparison with nymphal glands and for study of the histological changes in adult female corpora allata in relation to egg production.

The grasshoppers used in this study were reared as described by Pfeiffer (1945a). The corpora allata were dissected from freshly cut heads submerged in insect saline. For control purposes some of the corpora allata were dissected from heads not immersed in saline; they showed no difference from those dissected when submerged. The ages of the dissected grasshoppers were recorded in terms of the number of days that had elapsed since the preceding molt. The approximate length of the intermolt periods was learned from control nymphs of the same molting dates. When the corpora allata were dissected from adult females, the presence or absence of oviducal secretion and yolk was noted, the oocytes nearest the oviducts were measured, and the blood color was recorded. Several fixatives were tried. Of these, Susa's technique gave the best results and therefore was used almost exclusively. The glands were imbedded in paraffin. The sections, seven to eight micra in thickness, were stained with hematoxylin and eosin, toluidin blue and erythrosin, methylene blue and erythrosin, Masson's trichrome stain (hemalum, erythrosin and safranin) and Foot's modification of Masson's stain (Weigert's hematoxylin, Ponceau de xylidine, acid fuchsin and light green).

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[1] The author wishes to express her sincere thanks to the staff of Osborn Zoological Laboratory, Yale University, for the kind hospitality she has received while doing the present research. To Dr. I. W. Pfeiffer she expresses her gratitude for having introduced her to the field of insect endocrinology.

I owe to Dr. I. W. Pfeiffer the privilege of studying iron hematoxylin slides which were useful for the counting of chromosomes.

## Topography and Morphology

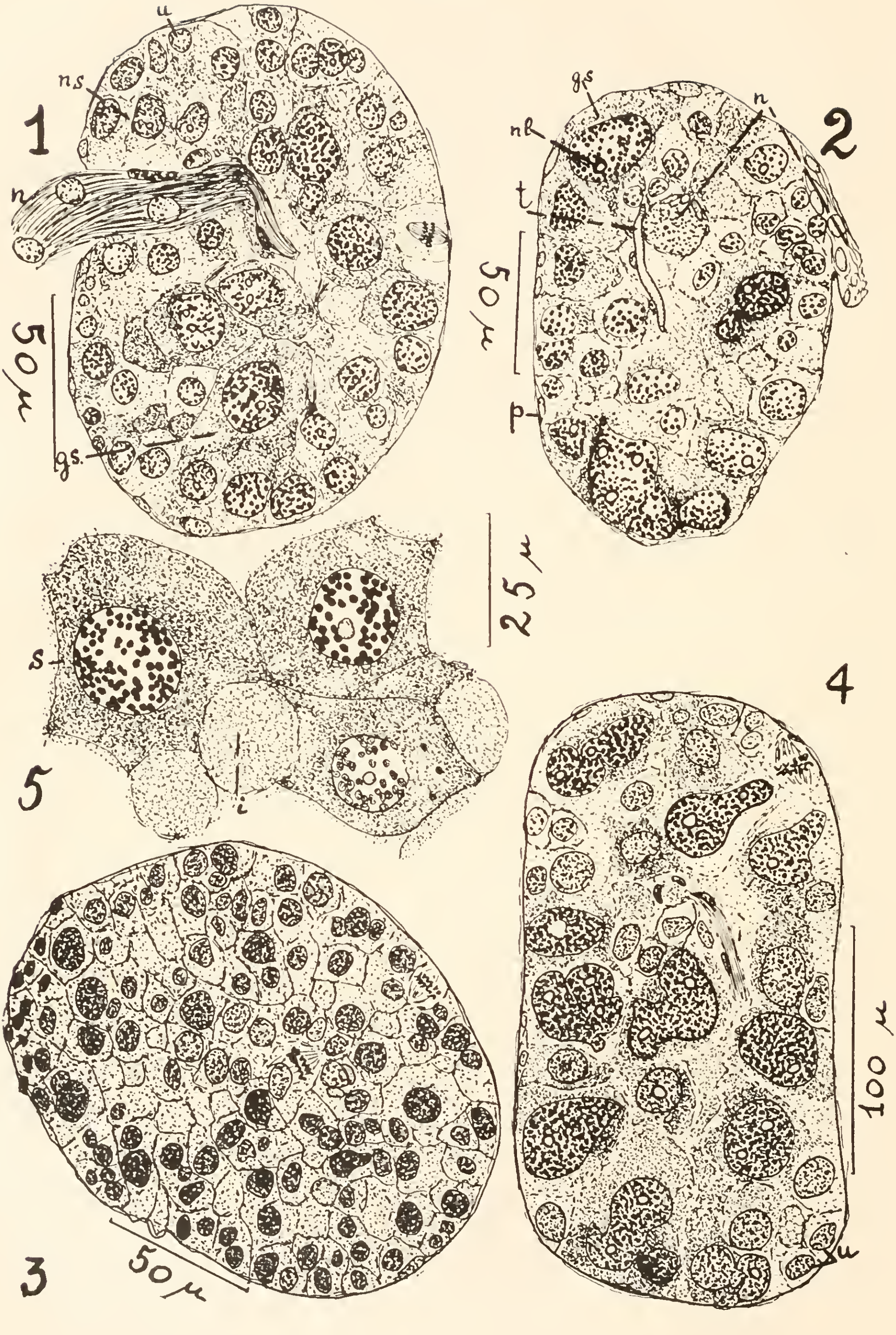
In *Melanoplus* the corpora allata are paired subspherical structures located ventro-laterally to the pharynx, just in front of the crop. The aorta has its anterior opening at about the same level. Therefore, the glands are suspended in a blood lacuna into which the contents of the dorsal vessel are discharged. They are innervated by the nervi corporis allati. There are no nerves emerging from the corpora allata. The nervus corporis allati enters through a slightly depressed point (the hilum, Fig. 1) and ramifies throughout the gland, branches reaching almost all of the glandular cells. Covering the gland is a delicate connective tissue sheath which penetrates the glandular tissue at the hilum and is in direct continuation with the perineurium that covers the nervus corporis allati. The nerve contains a few small cells with spherical nuclei different from the flattened perineurium cells; both kinds are found inside the gland. A trachea penetrates the organ with the nerve, and tracheal cells enter with it. This trachea branches abundantly, so that numerous tracheoles are to be found among the cells. Many fine tracheae may also be seen along the surface of the gland at points away from the hilum. They vary in number with the age of the animal, tracheae being more numerous on the surfaces of old or hypertrophied glands.

## Histology

*General.*—The structure of the corpora allata is fundamentally the same in males, females, nymphs and adults. However, sexual differences and developmental stage and age variations are sufficiently well defined that sex and approximate age of the animal can be estimated from the histological aspect of the corpora allata. Four types of cells may be recognized in the corpora allata: (1) connective tissue cells (of the outer sheath and the perineurium) and tracheal cells; (2) undifferentiated cells; (3) normal secretory cells; and (4) giant secretory or polyploid cells.

The undifferentiated cells (Figs. 1, 4 and 8) are small spherical or polyhedric cells, about 10 to 14 micra in diameter, with relatively large spherical nuclei. These cells have only a small amount of cytoplasm and do not contain secretory granules or vacuoles. They divide mitotically, exhibiting the greatest mitotic activity at the beginning of each developmental stage. However, they may occasionally be found in mitosis at other times during the nymphal or adult stages. Undifferentiated cells are present in the gland at all times.

PLATE I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The normal secretory cells (Fig. 1) may be distinguished from the undifferentiated cells only during active secretory stages of the gland. They vary in volume in accordance with the phase of the cycle of activity of the individual cells. They may become strongly polymorphic, at which time the nucleus is large, usually more or less lobulated, with abundant deeply staining chromatin; one nucleolus is always present. Under the conditions of fixation employed, the cytoplasm of the normal secretory cells is dense and acidophil. During active phases it has a granular appearance due to the presence of strongly acidophil granules. The granules appear first around the nucleus and gradually increase in quantity. When they have become numerous, intracellular vacuoles appear (Fig. 9). These vacuoles vary in size. They may become very large, occupying nearly the entire space at one side of the nucleus. In cells located near the periphery of the gland, the vacuoles are frequently situated on the side of the nucleus toward the center of the gland, under which conditions the cytoplasm in the outer portions of the cell may contain closely crowded acidophil granules. In some cases the content of the vacuoles appears as evenly dispersed granules. In others, particularly the larger ones, it has coagulated into a network. The staining reaction of the vacuolar contents is faintly acidophilic.

When intracellular vacuoles are numerous, vacuoles that are similar in appearance may also be seen between the cells (Figs. 5 and 8), suggesting that secretory material has passed out of the cells into intercellular spaces. At this time, also, particularly when the appearance of the gland suggests the most intense secretory activity, numerous fine lines which suggest "lines of flow" may be seen following somewhat radial courses from central regions of the gland to the periphery. They are especially noticeable in the outer portions of the gland (Fig. 9). When viewed with changing focus, they frequently appear to be between the cells since they are seen most distinctly before the nuclei and cytoplasm come into clear view. At the level of the nucleus the cytoplasm has the usual aspect of evenly

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### Explanation of Figures

1. Corpus allatum of fifth instar female nymph killed within 24 hours after molting. The four types of cells are recognizable. Acidophil granules are present in the secretory cells. No intra- or intercellular vacuoles have yet been formed. Mitoses are present. *gs*, giant secretory cell; *n*, nerve entering at the hilum; *ns*, normal secretory cell; *u*, undifferentiated cell.

2. Corpus allatum of fifth instar male nymph killed 8 days after molting. The secretory cells are beginning to decrease in size. Acidophil granules are scarce. A few intercellular vacuoles are still present. The giant secretory cells have polymorphic nuclei. They are more numerous in proportion to the number of normal secretory cells than in female corpora allata (compare with Fig. 1; see also Fig. 4). *gs*, giant secretory cell; *n*, nerve; *nl*, nucleolus; *p*, peripheral connective tissue cell; *t*, trachea.

3. Corpus allatum of sixth instar male nymph killed 2 days after molting. The different types of cells are hardly distinguishable. The cell membranes are distinct. Mitoses are numerous. No secretory material is present.

4. Corpus allatum of sixth instar male nymph killed 4 days after molting. Giant secretory cells with polymorphic nuclei are numerous. Acidophil granules are present near the nuclei. No intercellular vacuoles have as yet been formed. Occasional mitoses are still present. *u*, undifferentiated cells.

5. Three normal secretory cells from a corpus allatum of a sixth instar female nymph killed five days after molting. Intercellular vacuoles are present. Cell and nuclear membranes are indistinct in places. *i*, intercellular vacuole; s, acidophil granules.

dispersed granules and intracellular vacuoles. Sometimes, however, the acidophil substance in the outer regions of the peripheral cells may itself be disposed in lines perpendicular to the surface of the gland, thus contributing to the general aspect of outwardly radiating lines.

While the secretory granules are appearing, the nuclei of the normal secretory cells often become swollen and vacuolated, and the nuclear membranes become more delicate, until in some places no boundary between nucleus and cytoplasm can be distinguished (Figs. 5 and 8). However, acidophil granules were never found in the nucleus. When the nucleus acquires the swollen aspect, chromatin-like granules are usually found in the cytoplasm near it (Fig. 5). Mitoses were never found in secretory cells that had enlarged sufficiently to be distinguished from undifferentiated cells.

The giant secretory cells (Figs. 1, 2 and 8) are polyploid cells which arise from nuclear mitoses not followed by cytoplasmic division. The nucleus is two to four times larger than the nuclei of normal secretory cells. It is polymorphic, never spherical; its chromatin stains deeply, and it usually contains two to four nucleoli. When the nucleus is undergoing polyploid mitosis, the quantity of cytoplasm is about the same as that of normal diploid cells at rest, but thereafter it expands until the cytoplasmic volume is proportional to the chromatin content. As the cytoplasm becomes more abundant, the cell bodies tend to acquire an increasingly irregular shape. However, in every respect except size the giant secretory cells are similar to the normal ones. During the secretory phase acidophil granules appear in the same concentration as in normal secretory cells. Intracellular vacuoles develop. The nuclear membrane becomes gradually less distinct, and chromatin-like granules are frequently found in the cytoplasm. Most of the giant cells are located in the central portion of the gland, and during stages of intense secretory activity the gland, in sections through its center, may tend to have a stratified appearance (Fig. 8) with a central region containing giant cells and areas of dense cytoplasm, a more peripherally located region rich in intra- and intercellular vacuoles and an outer region in which the nuclei of secretory cells and undifferentiated cells are often arranged side by side to form a border. Due to cellular enlargement and the formation and growth of vacuoles, the gland expands during secretory activity.

Polyploid cells were found in which the chromosome number, counted in the equatorial plate, was between 90 and 100. The cells resulting from such mitoses must be octoploid since the normal diploid number for *Melanoplus differentialis* is 24 (King and Slifer, 1934). This large number of chromosomes was not very often encountered, but tetraploid cells are common in the corpora allata. In a few instances the number of polyploid mitoses in the corpora allata of recently molted nymphs was counted and compared with the number of giant cells in glands of the same nymphal stage which had passed the period of strong mitotic activity. This counting was most conveniently done on sixth instar corpora allata since here it is easy to know when the peaks of cell division and secretory activity are reached. A total of 5 polyploid mitoses, one of them octoploid, was counted in a series of 5 corpora allata from females examined 0 to 4 days after molting; therefore one in each gland. After the fourth day no more polyploid mitoses were found. From 3 to 8, or an average of 3.6 giant cells per gland, were found in females that had spent 5 to 7 days in the sixth stage.

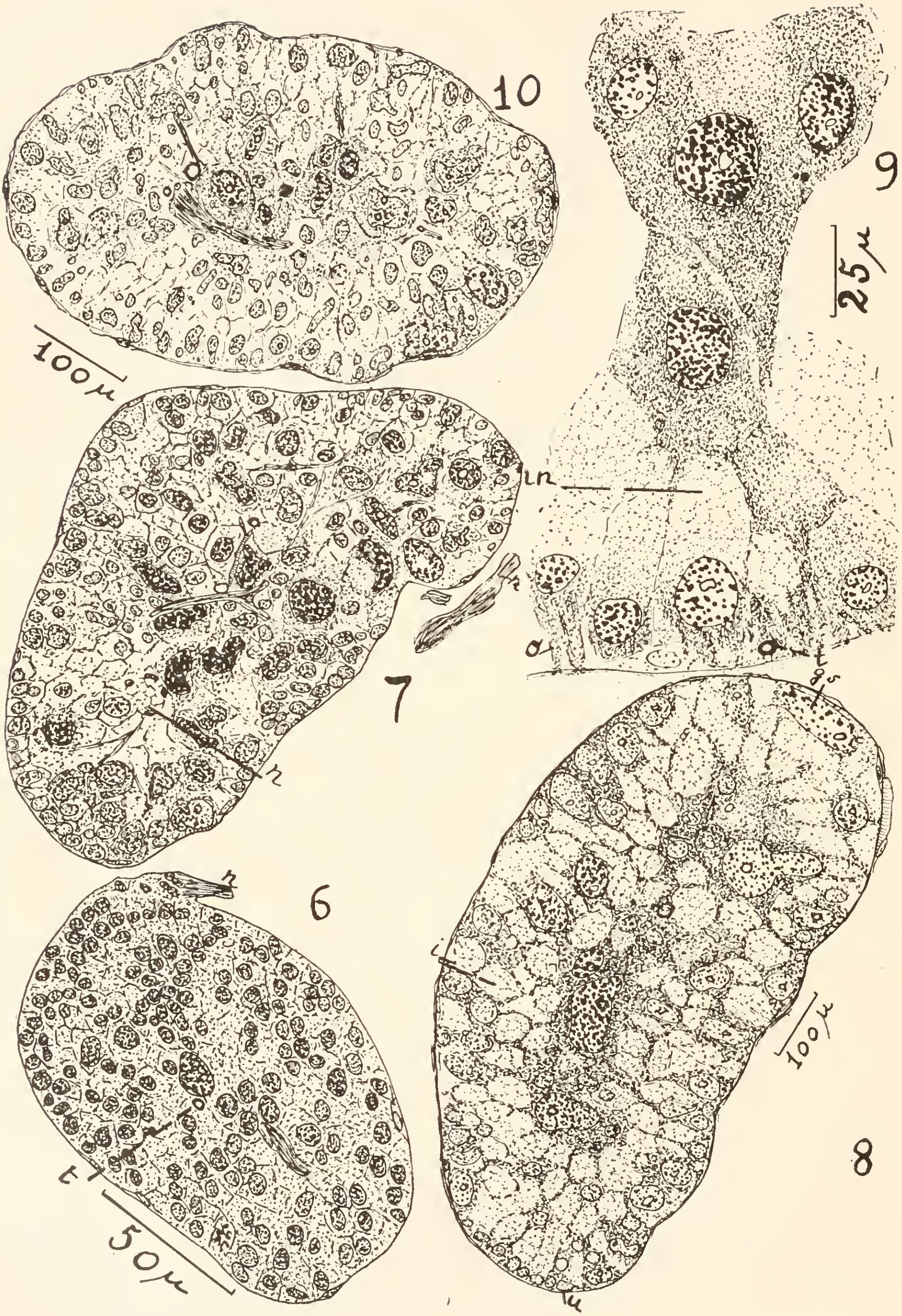
Polyploid mitoses were found only during the early part of each of the nymphal stages. In adults also, polyploid mitoses were most abundant during the days immediately following molting, but occasionally they were found later. The number of giant cells is progressively greater in each stage. Male corpora allata contain about the same number of giant cells as do the corpora allata of females of the same stage, but they are more prominent in the males because male corpora allata are slightly smaller and contain fewer normal secretory cells. The smaller size of the male corpora allata is presumably related to the fact that the males have a smaller total body size than do the females.

*Fifth instar corpora allata.* The corpora allata of newly molted fifth instar nymphs (Fig. 1) are subspherical, their major and minor axes measuring about 150 and 100 micra, respectively, in females, and about 120 and 100 micra in males. They contain both normal secretory and polyploid cells, as well as undifferentiated cells. The polyploid cells are readily distinguishable by their large nuclei. During the first two or three days of the fifth stage, in grasshoppers having a normal fifth stage intermolt period of seven to eight days, both diploid and polyploid mitoses occur. Mitosis continues up to the fourth to fifth days but less frequently. Acidophil granules are present in the secretory cells from the day the nymph enters the fifth stage until the next molt occurs. They are most abundant from the fourth to sixth days, the cytoplasm of the secretory cells at this time having an intensely acidophil reaction due to the closely crowded granules. Intra- and intercellular vacuoles are also abundant during the fourth to sixth days. The latter tend to increase in size after their first appearance, and the gland shows evidence of active release of secretory material from the fourth day on. By the seventh or eighth day (Fig. 2) both granules and vacuoles become less numerous, and the cells begin to decrease in size.

*Sixth instar corpora allata.* Just after the grasshoppers enter the sixth stage (Fig. 3) the corpora allata are subspherical, the length of their major and minor axes being around 180 and 130 micra in females and around 150 and 120 micra in males. At this stage all of the cells are similar in appearance, resembling undifferentiated cells. Both normal and polyploid mitoses are present, and immediately after molting the rate of mitotic activity is greatly accelerated, so that during the first three days, in grasshoppers having a sixth stage intermolt period of twelve to fifteen days, one to four and even five mitoses may be found in each section of eight micra. Mitoses are somewhat more numerous in females than in males, as might be expected since the male corpora allata are smaller and contain fewer cells.

On the second and third days after molting, although numerous mitoses are still present in the gland, the cytoplasm of many of the cells begins to increase in volume, and on the third and fourth days normal and giant cells are definitely distinguishable from undifferentiated cells. The cytoplasmic phenomena are the same in sixth stage corpora allata as described for fifth instar nymphs, but the period of secretory activity is briefer and the steps of the secretory process are more clearly defined. During the first two or three days of the sixth stage acidophil granules are absent from the gland. They begin to appear on the third or fourth day (Fig. 4). By the fifth day intra- and intercellular vacuoles are numerous and the "lines of flow" described earlier may be seen directed toward the periphery of the gland. The appearance of the gland at this time suggests that secretion is being released into the body cavity. However, from the seventh or eighth day

Plate II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until the end of the sixth stage the gland shows no signs of active production of secretion, and mitoses are infrequent. During this stage the giant cells shrink in both nuclear and cytoplasmic volume until it becomes impossible to tell them from normal diploid cells (Fig. 6).

*Adult female corpora allata.* In newly molted adult females the corpora allata are somewhat flattened bodies, usually roughly circular in outline. Their major and minor axes measure about 400 and 200 micra, respectively. At this time all of the cells are small and similar in appearance, as they were at the close of the sixth stage. However, immediately after molting, in grasshoppers developing at a rate such that oviducal secretion can be expected to appear on the seventh or eighth day of the adult stage, the corpora allata begin to enlarge rapidly, due to cell proliferation, to cytoplasmic expansion and to increase in nuclear volume (Fig. 7). During the first four or five days of the adult stage both normal and polyploid mitoses are present. They are found frequently until the first group of oocytes reach 0.6 mm. in length, but decrease in number during the period when the oocytes grow from 0.6 to 0.75 mm. By the time the production of oviducal secretion and yolk begins mitoses are rare, although they may occasionally be seen throughout the remainder of the adult stage, presumably to replace worn out cells.

Acidophil granules are lacking at the time the female enters the adult stage, but they may be present in some of the secretory cells by the second day after molting if development is rapid. Intra- and intercellular vacuoles may be present by the third or fourth day, with the gland having the appearance that release of secretion to the body cavity has begun by the fourth day. By the fifth or sixth day the gland may have the appearance of intense secretory activity, with the nuclei and cytoplasm greatly expanded, intra- and intercellular vacuoles large and the "lines of flow" strongly evident. The adult corpora allata usually show considerably greater expansion during active phases than is usual for the nymphal corpora allata.

After the secretory phase of the adult corpora allata has begun, it continues at a high level (Fig. 9) throughout the sexually active life of the female. The cytological and histological aspects remain the same and show no variation with blood color changes or with the egg development cycles. When the adult female

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EXPLANATION OF FIGURES

6. Corpus allatum of sixth instar female nymph killed 8 days after molting. Almost all of the cells look alike. Acidophil granules and vacuoles are absent. *t*, trachea.

7. Corpus allatum of adult female killed 2 days after molting. The four types of cells are readily recognizable. Acidophil granules are beginning to appear. *n*, nerve.

8. Corpus allatum of adult male killed 15 days after molting. The gland is at the height of secretory activity. Acidophil granules and vacuoles are present. The gland has a stratified appearance due to the distribution of the granule-filled cytoplasm and the vacuoles. *gs*, giant secretory cell with vacuolated nucleus, nuclear membrane indistinct in places; *i*, intercellular vacuoles; *u*, undifferentiated cells.

9. Portion of the outer region of a corpus allatum of an adult female killed 26 days after molting. The flow of secretory material toward the outside is shown at the periphery of the gland. *in*, intracellular vacuole; *o*, "lines of flow"; *t*, trachea.

10. Corpus allatum of an adult female killed 60 days after the last molt. The first signs of senescence are present. Acidophil granules are becoming scarce. Connective tissue is becoming more prominent both in the outer membrane and within the gland.

becomes old the amount of secretion in the corpora allata gradually diminishes, the outer membrane increases in thickness and the cytoplasm is stained with more difficulty (Fig. 10). Mitoses are absent and the gland decreases somewhat in size.

*Adult male corpora allata.* Except for their smaller size, the fewer normal secretory cells and the prominence of the giant cells, the corpora allata of adult males (Fig. 8) are histologically very similar to those of adult females developing at the same rate. The corpora allata of newly molted males are usually roughly circular in outline and somewhat flattened. The cells are small, resemble one another, and are devoid of acidophil granules. During the early part of the adult stage there is a period of cell proliferation and nuclear and cytoplasmic expansion. Both normal and polyploid mitoses occur frequently during the first three or four days after molting, then gradually decrease in number. As the cells expand, acidophil granules and then intra- and intercellular vacuoles appear. The corpora allata attain full secretory activity at about the same time in males as in females, and they show the same great expansion of the nuclei and cytoplasm during the active phase.

## DISCUSSION

Evidence from experimental studies supports the concept that in hemimetabolous insects (*Rhodnius*, Wigglesworth, 1934, 1936, 1940; *Dixippus,* Pflugfelder, 1937, 1937a, 1939; *Leucophaea*, Scharrer, 1946), as well as in holometabolous insects (*Bombyx*, Bounhiol, 1939), juvenile hormone is released by the corpora allata during the post embryonic stages which precede molts that are accompanied by the development of juvenile (nymphal or larval) characters but is absent or ineffective during the stage which precedes the production of adult characters and metamorphosis. The tissues are evidently stimulated to develop juvenile characters during a "critical period" in the nymphal (or larval) stage when juvenile hormone attains effective concentrations in the blood. That development of the grasshopper *Melanoplus differentialis* conforms to this principle has been suggested by the results of experimental studies (Pfeiffer, 1945) and is now further confirmed by the histological findings of the present investigation. It was seen in this regard that secretory material, presumably juvenile hormone, is elaborated in the corpora allata throughout the greater part of the fifth (next to last) nymphal stage and is most actively released during a period that begins around the middle of the stage and continues until shortly before molting. The "critical period" during which the insect is caused to develop the nymphal characters that appear at the end of the fifth stage may, therefore, be supposed to occur during the latter half of this stage.

On the other hand, the corpora allata of *Melanoplus* also show evidence of secretory activity in the sixth stage, a fact which suggests that juvenile hormone is again being produced. If this is true, it must be concluded that the hormone released in this stage is ineffective in inducing the development of nymphal characters, since metamorphosis occurs at the end of the stage. A reason for such ineffectiveness is suggested by the fact that the entire period of elaboration and release of secretory material in the sixth stage is brief; it does not begin until the second or third day after molting and is completed around the middle of the stage. It would therefore seem probable that the amount of hormone produced is inadequate, or its release may not take place at the proper time to prevent metamorphosis. There is also the possibility, suggested by evidence from other insects (*Drosophila,* Bodenstein, 1943; Vogt, 1943; *Leucophaea,* Scharrer, 1946), that the tissues may be less responsive to juvenile hormone in the sixth than in earlier stages, a condition which would further decrease the effectiveness of such amounts of hormone as are produced. In regard to these possibilities, Pfeiffer (1945) has shown that metamorphosis can be delayed in *Melanoplus,* and nymphal characters caused to appear at the end of the sixth stage, by supplying additional amounts of corpus allatum hormone.

Wigglesworth (1934) has also described histological evidence of secretory activity in the corpus allatum of *Rhodnius* during the last (fifth) nymphal stage but was unable by experimental means to detect the presence of juvenile hormone in the blood. On the basis of recent experiments (1947) he has postulated that the corpora allata of old fifth stage nymphs or young adults of *Rhodnius* cause removal of juvenile hormone from the blood. No evidence of a similar function has been observed in the histology of the corpora allata of *Melanoplus.* However, that hormone from the corpora allata may fall to low concentrations in the blood or disappear at the end of the last nymphal stage in this insect, and that it does not regain high levels until about the time the influence of the corpora allata on adult functions (Pfeiffer, 1939, 1945a) is manifest, is suggested by the histological evidence. The corpora allata, it was seen, show no evidence of secretory activity during the last six or seven days of the sixth stage, or on the day of entering the adult stage, but achieve the histological appearance of strong secretory activity shortly before the production of oviducal secretion and yolk begins. Pfeiffer (1945a) has shown in this connection that metabolic conditions in females of *Melanoplus* during the early part of the adult stage are like those continuously in effect when the corpora allata have been removed, but change when there is sufficient hormone in the blood to induce the production of oviducal secretion and yolk.

It is of interest that after the corpora allata have attained high levels of secretory activity in adult females, they continue functioning at such levels, showing no histological evidence of cyclic changes related to the egg production cycles. Joly (1945) likewise found no histological evidence of intermittent activity of the corpora allata of *Dytiscus* but reasoned from experimental evidence that the corpora allata function cyclically in adults of this insect. Also of interest is the fact that the corpora allata of adult males of *Melanoplus* show evidence of as intense secretory activity per unit of glandular tissue as do those of adult females, suggesting that the concentration of hormone in the blood may be maintained at similar levels in both sexes. The smaller size of the corpora allata in the males seems to be related to the smaller total body size of the males rather than to a functional sexual dimorphism. Considering that the corpora allata have a number of functions related to reproduction in adult females of *Melanoplus* (Pfeiffer, 1939, 1945a), but have so far not been found to have comparable importance in males, it could have been expected that they would show a lower level of activity in the males. The histological evidence would therefore seem to support the idea, which has been gaining increasing favor (Pflugfelder, 1938a; Day, 1943; Pfeiffer, 1945a), that the various functions of the corpora allata (see reviews by Hanström, 1939; Scharrer, 1941, 1948; Bodenstein, 1942; Joly, 1945a; Mendes, 1947) are accomplished, in part at least, through effects on metabolic processes rather than through specific actions on the different tissues involved.

In regard to whether the corpora allata secrete different hormones to perform different functions, it can only be said from the present observations that no evidence could be detected histologically that more than one hormone is produced either simultaneously or in the different stages. On the other hand, the fact that the corpora allata of adult grasshoppers may assume an appearance of more intense secretory activity than is seen in nymphs presents the possibility that the concentration of hormone from the corpora allata may reach higher levels in the blood of the adults and that this may have some significance in connection with the different functions of the corpora allata in nymphs and adults. It has been suggested (Bodenstein, 1943; Scharrer, 1946) that differences in hormone concentration and responsiveness of the tissues may play an important part in this connection.

The corpora allata have been described, both topographically and histologically for a large number of insects, the most comprehensive study in this regard having been made by Nabert (1913). References to the many recent descriptions may be found in papers by Hanström (1942) ; Poulson (1945) and Mendes (1947). The histological characteristics of the corpora allata of *Melanoplus* agree in a number of respects with those described for other insects. Among these may be mentioned: the acidophil reaction of the secretory granules; irregular shape of the cells and nuclei under certain conditions; abundance of chromatin; and the presence of large nucleoli. The occurrence of fine fibrils or striations which may constitute the same phenomenon as that described as "lines of flow" in the present study has been reported to occur in the corpora allata of termites (Pflugfelder, 1938) and in *Gryllotalpa* (De Lerma, 1932). Vacuoles are usually mentioned as a characteristic of active corpora allata. However, Wigglesworth (1934) did not find vacuoles in the corpus allatum of *Rhodnius* when the gland was active, but instead saw them between the cells when the latter had shrunk during a period of inactivity. In *Melanoplus*, it was seen, intercellular vacuoles were present at the height of secretory activity.

It is a general characteristic of the corpora allata that they expand during secretory activity and shrink during inactivity. The expansion may be due simply to enlargement of the cells or, as has been seen in *Melanoplus*, to both cellular expansion and the presence of large vacuoles. There may also be differences in the fundamental size of the gland which are related to the body size of the insect and are based on differences in the number of cells in the gland. In *Melanoplus*, it was seen that the corpora allata are larger and contain more cells in the females than in the males, which in all stages are, on the average, smaller individuals than are the females. The corpora allata also becomes progressively larger in each developmental stage. In agreement with other hemimetabolous insects (*Rhodnius*, Wigglesworth, 1934; *Dixippus*, Pflugfelder, 1937), the latter growth takes place during a period of cell proliferation which occurs at the beginning of the developmental stages. *Rhodnius* differs from *Melanoplus* and *Dixippus*, however, in that there is no proliferative phase at the beginning of the adult stage (Wigglesworth, 1934).

The cells of the corpora allata have generally been described as approximately alike, the principal variations being those shown by all of the cells of the gland in relation to activity and inactivity. However, in the corpora allata of fourth instar nymphs of *Rhodnius*, Wigglesworth (1934) distinguishes between centrally located cells which become swollen and acidophil during the critical period, and peripherally located, dividing and growing cells which do not become acidophil. These differences suggest a distinction between undifferentiated cells and secretory cells similar to that seen in *Melanoplus*. De Lerma (1932) found giant cells in *Gryllotalpa* which may be comparable to those observed in *Melanoplus* and which he describes as arising from the fusion of several cells. In *Melanoplus,* on the other hand, it was seen that the giant cells are the result of polyploid mitoses. Since these mitoses occur during the early part of each developmental stage, when mitotic activity is most intense and when the gland is entering an active secretory phase, it seems unlikely that they represent a degenerative condition. Instead, the fact that they have a large volume of cytoplasm in which is elaborated secretory material that cannot be distinguished histologically from that produced by the normal secretory cells suggests that they may be a means of increasing the secretory effectiveness of the gland. It is of interest that the giant cells make up a greater proportion of the gland in males then in females.

It seems probable that the polyploid chromosome content of the giant cells is the result of two or more mitoses of the nuclei of undifferentiated cells taking place in rapid succession. This hypothesis is supported by the fact that the volume of cytoplasm in these cells remains small until division of the nucleus is completed, suggesting that the nuclear divisions are not separated by periods of cytoplasmic expansion. The normal secretory cells, on the other hand, evidently arise from normal diploid division of the undifferentiated cells. This process occurs principally during the proliferative period in each developmental stage, but may occur at other times. Since undifferentiated cells are present in the gland at all times they constitute a reserve from which additional cells may be produced during the time the gland is in the secretory phase. They are also carried over from one stage to the next and are probably, in part at least, the cells in which the new wave of mitotic activity originates. It is possible, however, that the normal secretory cells may also undergo mitosis when they are reduced in size during inactive periods.

## Summary

1. The corpora allata of the grasshopper *Melanoplus differentialis* were studied histologically to determine whether these glands show changes related to their functions in the control of nymphal development and the production of ripe eggs and oviducal secretion.

2. Four classes of cells were recognized in the corpora allata of this insect: a, connective tissue and tracheal cells; b, undifferentiated cells; c, normal secretory cells; and d, giant secretory or polyploid cells.

3. The undifferentiated cells are small cells which divide mitotically and do not contain secretory granules or vacuoles. They show the greatest mitotic activity at the beginning of each developmental stage but occasionally divide at other times.

4. The glandular cells, both normal and polyploid, show characteristic changes related to secretory activity and inactivity which are fundamentally the same in nymphs and adults and in both sexes. During inactive phases they are reduced in size and resemble undifferentiated cells. During active phases they increase in cytoplasmic and nuclear volume, the cytoplasm becomes filled with strongly

acidophil granules, then intracellular and finally intercellular vacuoles appear. At the height of secretory activity the appearance of the gland suggests that materials are flowing from central regions of the gland to the periphery.

5. In the fifth nymphal stage, at the end of which nymphal characters are produced, secretory material is elaborated throughout the intermolt period; active release of secretion occurs during the latter half of the stage. In the sixth stage, which terminates with the production of adult characters and metamorphosis, the glands show histological evidence of activity only during a brief period which begins on the second or third day after molting and ends around the middle of the stage.

5. The corpora allata are inactive on the day the grasshopper enters the adult stage, but the histological changes which lead to the production and release of secretory material begin immediately thereafter. Full secretory activity is achieved in the corpora allata of adult females shortly before the onset of the production of oviducal secretion and yolk. It is reached at an equivalent time in adult males.

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# INFLUENCE OF GENETIC ENVIRONMENT ON THE REDUCTION OF BRISTLES BY THE DICHAETE GENE IN DROSOPHILA MELANOGASTER

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## INTRODUCTION

The interaction and influence of one gene upon another is one of the methods employed in the study of gene action. The influence on the Dichaete gene can be readily measured, for this gene removes the bristles in the region of the presutural bristles. Thus, a bristle is either present or absent, and the quantitative effect can be accurately determined. Various mutations were brought into combination with the Dichaete gene, and the effect on the number of bristles was recorded.

Gene action has been analyzed by studying the effects of different dosages of mutant genes upon the phenotype (Stern, 1929, 1943; Schultz, 1935). Another method is to prolong larval life by means of low temperatures, genes or starvation (Green and Oliver, 1940; Green, 1946; Dunn and Coyne, 1935). High temperatures, which shorten larval life, cause a decrease in the number of bristles in the mutant Dichaete (Plunkett, 1926), and alter the phenotype in other Drosophila mutants (Stanley, 1931; Child, 1935; Harnly, 1936). Neel (1941) and Sparrow and Reed (1940) reported on the interaction of mutants that affect the chaetae of *D. melanogaster*. Stone (1947) used the effect of several mutants on Dichaete in an attempt to discover whether differences existed in reciprocal crosses.

An attempt was made to find a relationship, if one existed, between the number of bristles of the Dichaete mutant and the following mutations: Bar (*B*), black (*b*), Curly (*Cy*), eosin ($w^e$), eyeless (*ey*), heldout (*ho*), Lobe² ($L^2$), vestigial (*vg*), white (*w*), and yellow (*y*). The above mutants were picked for two reasons: one is that they were not in the third chromosomes, and that the mutations by themselves had no apparent effect on the thoracic bristles.

## METHODS

The Dichaete gene and the above mutant genes were placed in a common genetic background by the following method. The highly inbred Dichaete stock (inbred brother and sister for nine generations) was crossed to the mutants and then the mutants were extracted. This procedure was repeated eleven times for each mutant, and according to the formula developed by Bartlett and Haldane (1935) about 14 to 18 units on either side of the mutant gene remained heterozygous.

Plunkett (1926) reported that the bristle number of Dichaete flies was influenced by poor food conditions, high temperatures and crowding. Since the environment has a definite effect on bristle number, all matings were made under uniform conditions. The flies were raised at 24° C., and the same amount of cornmeal-agar formula was used in each one-half pint bottle. Pair matings were used, and the parents were removed at the end of four days.

Bristles were counted on the right side of the scutellum, mesonotum, and on the right humerus. There are a total of twenty-six thoracic bristles, and the bristle count of the right half of the wild fly was then thirteen. The mean bristle count for the Dichaete stock was 10.634 ± 0.306 (♀♀) and 10.789 ± 0.147 (♂♂). The presutural, anterior supra-alars, and anterior dorsocentrals were the bristles that were usually missing in the Dichaete stock.

Table I

| Genotype | Mean number of thoracic dorsal bristles of one-half of the fly (right side) | | | |
|---|---|---|---|---|
| | ♀♀ | No. | ♂♂ | No. |
| *D/+* | 10.634 ± 0.306 | 592 | 10.789 ± 0.147 | 493 |
| *D/+ B/+* | 10.325 ± 0.896 | 623 | 10.394 ± 0.642 | 601 |
| *D/+ b/b* | 10.723 ± 0.711 | 246 | 10.641 ± 0.541 | 315 |
| *D/+ Cy/+* | 8.724 ± 0.742 | 419 | 8.970 ± 0.866 | 486 |
| *D/+ ey/ey* | 10.774 ± 0.419 | 283 | 10.861 ± 0.302 | 206 |
| *D/+ ho/ho* | 12.818 ± 0.149 | 336 | 12.444 ± 1.148 | 376 |
| *D/+* $L^2/+$ | 10.191 ± 1.391 | 484 | 10.889 ± 0.168 | 479 |
| *D/+ vg/vg* | 11.250 ± 0.521 | 229 | 12.253 ± 0.636 | 288 |
| *D/+ w/w* | 10.841 ± 0.413 | 396 | 10.718 ± 0.662 | 384 |
| *D/+* $w^e/w^e$ | 10.639 ± 0.523 | 344 | 10.392 ± 0.493 | 369 |
| *D/+ y/y* | 10.614 ± 0.614 | 248 | 10.743 ± 0.714 | 293 |

The Dichaete stock was crossed to each of the above mutants. Both ♀ and ♂ Dichaete flies were employed. The thoracic bristles were counted in the $F_1$ in the case of *B*, *Cy*, $L^2$ and in the Dichaete males containing *w*, $w^e$, and *y* genes. The other combinations were counted in the $F_2$.

## Results

In Table I the mean (or average) and the standard deviation of the mean of the thoracic dorsal bristles (right side) of *D/+* and other genotypes are presented. In the Dichaete Curly cross, the *D/+ Cy/+* flies had a lower mean (8.724 ± 0.742♀♀). This is a highly significant difference compared to mean of 10.634 ± 0.306♀♀ of *D/+*, for the difference is more than three times the probable error. The genes *vg* and *ho* significantly increased the mean of the bristle number to 11.250 ± 0.521 and 12.818 ± 0.149 respectively for females. The *B*, *b*, *ey*, $L^2$, *w*, $w^e$, and *y* genes had no influence upon the thoracic bristles when in combination with the *D* gene. The males had a slightly higher mean number of bristles than the females.

## Discussion

The action of the Dichaete gene had been postulated by Plunkett (1926) to remove bristles in a direction radiating outward from the presutural bristle. A catalyst which decomposes another bristle-forming catalyst was the manner in which bristles were supposed to be removed. Neel (1941) questioned the existence of the above diffusion hypothesis in scute, but in counting the bristles of Dichaete flies and Dichaete-mutant combinations it was evident that a gradient existed. In flies with 12 bristles on the right side, it was always the presutural bristle that was missing. In the *D/+ Cy/+* combination, the bristles anterior notopleural, anterior supra-alars, anterior dorsocentral, and upper humerals were the bristles that were usually missing. There was a gradient present in the direction of the anterior supra-alar bristle. It is possible that the bristle-removing substance produced by the Dichaete gene is different from that found in scute, although they both remove bristles.

The Dichaete gene evidently produces substances directly or indirectly that destroy or inhibit the bristles. Now this system is disrupted by high temperatures which evidently speed up a reaction or reactions which produce more bristle-destroying or inhibiting substance, and thus a fly with fewer bristles is the result. The above system of removing or limiting the number of bristles is thrown out of balance by poor food conditions and low temperatures which evidently slow down the reactions and permit the bristle-destroying catalyst or bristle-inhibiting substance to act for a longer time, again producing flies with fewer bristles.

One encounters some difficulty in using the above scheme in explaining the effect of genes upon the Dichaete bristle system that neither increases nor decreases the life cycle of the flies. The *Cy* gene reduces the number of bristles in a Dichaete fly, and yet it neither speeds up nor slows down the length of time the individual insect spends in egg, larval, or pupal stages. Evidently the *Cy* gene produces in some way a larger amount of the bristle-destroying or inhibiting substances or may cause these substances to begin reacting earlier and to last longer.

The Curly gene turns the tips of the wings upward, and is a small inversion in the second chromosome. *D* is also a result of an inversion, and besides removing bristles also spreads the wings apart at a 45° angle. This may be a coincidence, or there may be a relationship between wing mechanism and bristle formation. The presence of genetic modifiers in the *Cy* inversion has to be considered. However, the method of inbreeding and selection would result in the *Cy* flies all having the same chromosome rearrangement. If the *Cy* flies used contained different genetic modifiers, the *D/+ Cy/+* flies would have a greater standard deviation of the mean (would be more variable) than the *D/+* flies. This difference is not significant (Table I).

The *vg* and *ho* genes are wing mutants, and they increase the length of time spent in the larval stage. One would expect a low bristle number according to Plunkett's hypothesis (1926), but that is not the case (Table I). It may be the interruption of the wing forming mechanism that influences the removal of bristles by the Dichaete bristle destroying substance. There appear to be several unrelated factors which can cause a variation in the number of bristles reduced by the Dichaete gene. Thus, the bristle destroying substance does not act simply upon a bristle forming substance. One cannot say that a substance *A* produces *B* which is a precursor of a bristle destroying substance *C* which acts upon a substance *D* which in turn produces bristles. There are probably more than three substances in the chain, and there is evidence, because of diverse environmental and genetic modifiers, that numerous side chain reactions exist that influence the step by step production of the bristle destroying or inhibiting substance. It is a complex process in that these variable factors may work at different times or several together at various times. Temperature, food conditions, other seemingly unrelated genes, and chromosome arrangements play an important role in the action of the Dichaete gene.

## SUMMARY

A highly inbred Dichaete (*D*) stock of *D. melanogaster* was crossed to isogenic stocks carrying various mutations, which by themselves had no apparent effect upon thoracic bristles. The Curly gene (*Cy*) in combination with *D* decreased the average number of one half the thoracic bristles from 10.634 ± 0.306(♀♀) to 8.724 ± 0.742. The *Cy* gene is an inversion on the second chromosome, and it aids the *D* gene, which is on the third, in the removal of a larger number of bristles. Vestigial (*vg*) and heldout (*ho*) increase the bristle number to 11.520 ± 0.521 and 12.818 ± 0.149 respectively. *Vg* and *ho* increase the larval life, and disrupt the production of a wild type wing.

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# STUDIES ON ARTHROPOD CUTICLE. II. ELECTRON MICROSCOPE STUDIES OF EXTRACTED CUTICLE [1, 2, 3]

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In previous work with the electron microscope it was found that sections of cockroach cuticle showed alternating denser and less dense laminae (Richards and Anderson, 1942a). Following treatment with hot alkali solutions this density differentiation was lost. Several possible explanations were suggested in the above paper, the two chief ones being that either a localized heavy component (e.g., protein) in the dense laminae was removed, or that the alkali treatment not only removes certain components but also causes or is followed by a redistribution of the remaining components (at least principally chitin). The x-ray diffraction studies of Fraenkel and Rudall (1940, 1947) suggest that the latter explanation is more probably correct. The present studies were undertaken to clarify this point further and to evaluate the use of isolated cuticles in permeability studies.

In general, we have found that any treatment leading towards chitin purification alters the membrane structure extensively. It follows that the extraction method of analysis has little value for a study on localization of components in the cuticle, that the cuticle cannot be viewed as a fixed framework of chitin micelles in which the other components are embedded, and that purified chitinous membranes, such as used by Yonge (1936) and Alexandrov (1935), are without biological significance for studies on the permeability of arthropod cuticle although normal isolated cuticles may give valid data. Unexpected variations between purified chitin membranes from different sources were found; the significance of these variations is discussed.

## MATERIALS AND METHODS

In our electron microscope studies on arthropod cuticle we have found it most satisfactory to perform the bulk of the experimental and observational work on readily prepared thin membranes. Subsequently results are checked by examination of other cuticular membranes and, when necessary, with sectioning and other ancillary methods. Accordingly the bulk of the work recorded in the present paper was done with large tracheae from a cockroach (*Periplaneta americana*). These tracheae contain chitin as well as protein, polyphenol and lipid components. Data obtained with cockroach tracheal membranes were then checked with crop linings,

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[2] The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army and the University of Minnesota. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

[3] Acknowledgment is due to the Electron Microscope Committee of the University of Minnesota for making the instrument available to us, and to Miss Virginia Kletzin for technical assistance.

rectal linings, soft general body cuticle, wing membranes, various types of scales and setae, and other tracheal membranes including ones consistently negative to chitin tests. The work recorded in the present paper was restricted to membranes sufficiently thin for direct electron micrography.

Normal structure was determined from membranes dissected out in saline solution, rinsed briefly in distilled water, and then placed on electron microscope screens and air-dried (Richards and Anderson, 1942 a, b, c; Richards and Korda, 1947, 1948). Tests on series of specimens treated with distilled water for periods ranging from several minutes to several hours at room temperatures showed no significant differences as far as can be detected with electron micrographs; if the brief rinse in distilled water necessary to clean the surface of the tracheal membranes has any effect on the membrane itself we were not able to detect it (see also under water extraction experiments in main section of this paper). The larval cuticle of mosquitoes and membranes from the gut were wiped gently with a soft brush to facilitate cleaning. The mosquito wing membranes were mounted directly without any treatment except the indicated experimental treatments. The membranes used in the present studies are reasonably large; accordingly they were placed directly onto the screens used in electron micrography (i.e., no supporting membrane of collodion, formvar, etc.) and the membrane under consideration is the only membrane in the field.

Experimentally treated membranes were handled in the same manner except for receiving the indicated treatments. In all experiments, a piece of membrane was removed from the animal, divided into two or more pieces, one of which was always mounted as control for the other piece or pieces which were given treatments. All tests recorded were run in duplicate, and many of them were repeated.

In electron microscope work on arthropod cuticle it is always necessary to consider the possibility of instrumental errors, especially the destructive effects of electron bombardment and of heat produced thereby (Richards and Anderson, 1942a; von Borries and Glaser, 1944). The more gross effects recorded in the present paper can be readily seen by dark-field microscopy or phase-contrast microscopy in membranes still in the solutions used. They are therefore not produced by either the drying or the electron bombardment—although it is quite possible that some of them may be augmented by these factors. The similar appearing but more minute effects cannot be observed directly with a light microscope but it seems reasonable to presume that those shown in the present paper are, like the gross effects, produced by the chemical treatment because birefringence values do not change significantly on drying.

As is generally known, one may remove from insect cuticle (a) the water, (b) the lipids, (c) the water-soluble protein, or (d) presumably all the components other than chitin.[4] No method is known for the removal of chitin without the simultaneous complete dissolution of the membrane. Since electron micrography must be performed in a vacuum, it has not been possible to study hydrated membranes. Treatments used were selected from the literature, and were chosen to cover the various methods that have been used for the removal of certain components, especially those used for the purification of chitin.

[4] Calcium salts which make up so large a part of many crustacean cuticles are absent from almost all insect cuticles (absent from all of those studied by us).

*Table of Treatments Employed*

| Agent | Strength | Temp. (°C.) | Time | Type of cuticle |
|---|---|---|---|---|
| KOH | 5% | 20 | 6 + 10 days | C.T. |
| | 5% | 100* | 1 day | C.T., M.W. |
| | 20% | 25 | 2 days | M.L.C. |
| | 20% | 65 | 2 days | M.L.C. |
| | 20% | 85 | 4 hours | C.T. |
| | 20% | 100* | 1 day | M.W. |
| | 40% | 100* | 10 minutes | L.P.M. |
| | conc. (= ca. 60%) | 160* | 15 min. to 2 hrs. | C.T., C.H.G., C.C., Ce.S., L.C., M.W., M.L.C., (H.B.A.S.)† |
| NaOH | 5% | 20 | 2–12 days | C.T., M.L.C., H.C., M.L.T., Ce.S. |
| | 5% | 65 | 6 + 10 days | C.T. |
| | 5% | 100* | 5 + 9 days | C.T. |
| | 10% | 100* | 1 day | (H.B.A.S.) |
| | 20% | 85 | 4 hours | C.T. |
| HCl | 5% | 100* | 1 day | C.T. |
| Pepsin (+ HCl) | 10% | 36 | 1 day | C.T., L.C., L.P.M., H.B.A.S. |
| Diaphanol‡ | full | 25 | 1, 5, 10 + 41 weeks | C.T., C.C., (H.B.A.S.) |
| $KMnO_4$ | 0.1% | 20 | 2 days | C.T. |
| $H_2O_2$ | 30% | 20 | 2 days | C.T. |
| $CHCl_3$ (through acetone) | C.P. | reflux | 5 min. to 2 hrs. | C.T., H.B.A.S. |
| $CHCl_3$ then pepsin | as above | as above | $CHCl_3$ for 2 hrs.<br>pepsin for 1 day | C.T., H.B.A.S. |
| Aerosol OT§ | 0.02% | 25 | 1 day | H.B.A.S. |
| Isotonic salt sol. | — | 25 | 2–7 days | C.T., H.B.A.S. |
| Distilled water | — | 25 | few min. to 2 weeks | C.T., H.B.A.S., M.L.C. |
| | — | 30 | 1 + 2 days | H.B.A.S., M.W. |
| | — | 60 | 1, 7 + 14 days | C.T., M.W. |
| | — | 65 | 1, 2 + 7 days | H.B.A.S., M.L.C. |
| | — | 100* | 1, 7, 14 + 28 days | C.T., H.B.A.S., M.W., M.L.C. |
| | — | reflux | 5 days | C.T. |

* In sealed glass ampoules.

† Parentheses indicate that the membrane was completely destroyed by the treatment.

‡ Saturated solution of chlorine dioxide in 50 per cent acetic acid.

§ Di octyl sodium sulfo succinate.

C.C. = Cockroach (*Periplaneta americana*), membrane lining crop.
C.H.G. = Cockroach, membrane lining hind gut.
C.T. = Cockroach, trachea (large trachea extending anterior from prothoracic spiracle to head).
Ce.S. = Centipede, sensillae (Richards and Korda, 1947).
H.B.A.S. = Honey bee, abdominal air sac (parentheses indicate destroyed).
H.C. = Housefly, membrane lining crop.
L.V. = Blowfly (*Lucilia illustris*), membrane lining crop.
L.P.M. = *Lucilia*, peritrophic membrane.
M.L.C. = Mosquito (*Aedes aegypti*), larval cuticle of abdomen.
M.L.T. = Mosquito, larval tracheae.
M.W. = Mosquito, wing membrane.

The preparations given lengthy treatments and those treated at higher temperatures were placed in sealed glass ampoules. Most of the hydroxide treatments were given in pyrex glass but a few were run in tubes of fused silica. The preparations from membranes treated with alkali must usually contain a trace of alkali because this is not completely washed out of cuticle by rinsing in water (or even prolonged soaking). Probably more alkali remains in those preparations washed in alcohol. Full removal of the alkali by soaking in one per cent HCl followed by washing in distilled water does not alter the electron microscope picture; accordingly we have usually ignored this trace of alkali that may remain in the preparation (Figs. 5–6 and 10–12 show preparations treated with HCl to remove all the alkali, while Figs. 7–9 were not washed in acid solution). The effects of washing in various ways is discussed in the next section. Diaphanol is an oxidizing agent prepared by saturating a 50 per cent solution of acetic acid with sulfur dioxide; it can be washed from cuticle with water but we used the supposedly gentler treatment (recommended by Koch, 1932) of transferring through 50 per cent, 30 per cent and 10 per cent acetic acid to water, then washing in one per cent $NaHSO_3$ at room temperature or 2 per cent $Na_2S_2O_3$ at 97° C., and finally washing again in water. Preparations treated with potassium permanganate were washed first in water, then in 0.1 per cent $NaHSO_3$, and then again in water. Specimens refluxed with chloroform were transferred from water to chloroform through acetone, and back to water through acetone; they were dried from water. For extraction with aqueous media, double distilled water was used, the second distillation being made in a pyrex glass still.

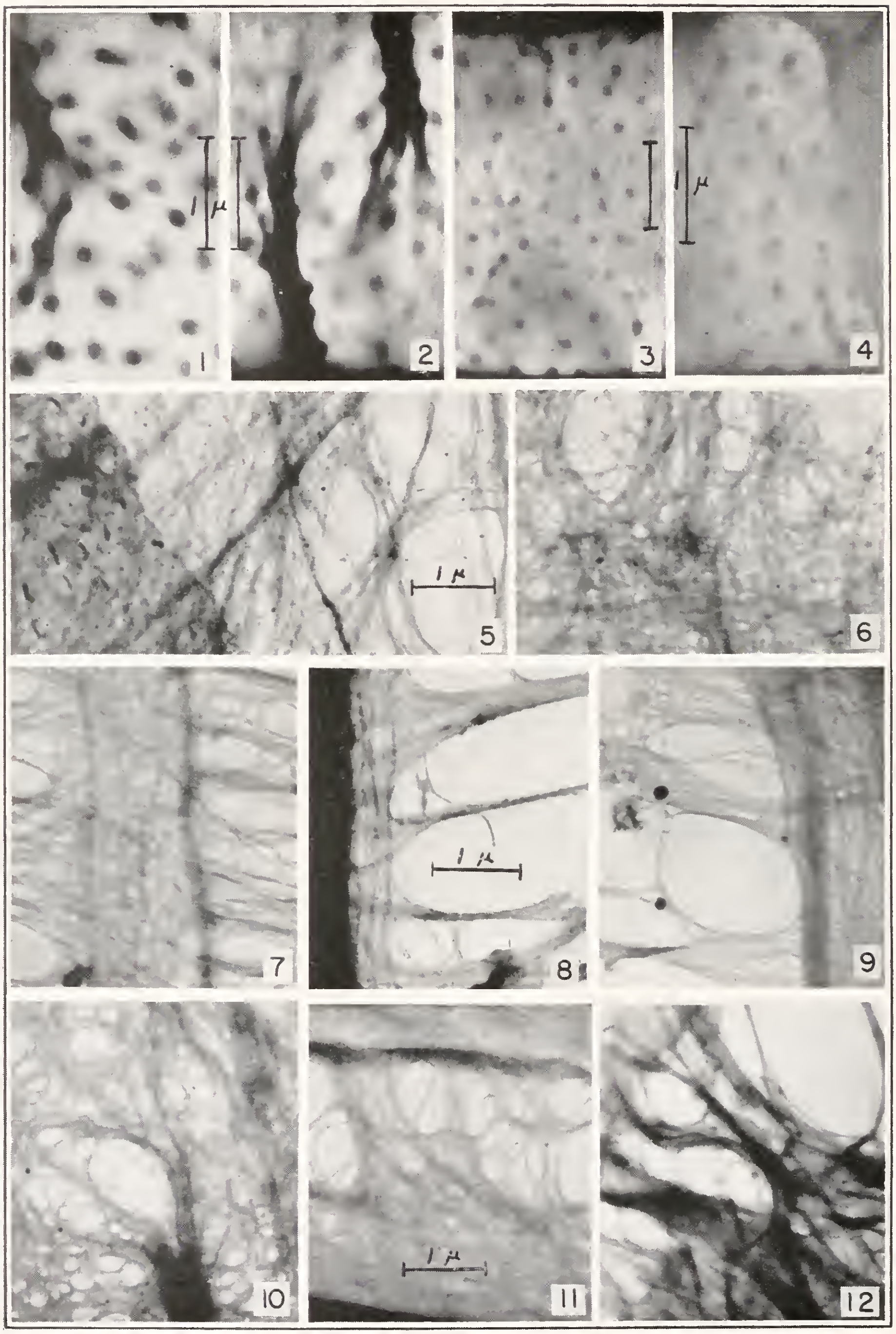
The electron microscope used was an RCA model EMU which uses an accelerating potential of 50 kilovolts. Most of the present work was done with the standard instrument as received from the factory. More recent pictures (those bearing numbers above 300) were taken after modifying the instrument by installing a saturation emission type electron gun and compensating the objective lens and after introducing the recently developed operational refinements to increase resolution (Hillier and Ramberg, 1947). Electron micrographs were made at an initial magnification of 6,000–10,000 and photographically enlarged to the size used for reproduction. Resolution in most of the figures of thin membranes presented is better than 0.01 $\mu$; in some of the best it is of the magnitude of 0.005 $\mu$; resolution in thicker membranes (wings, mosquito cuticle) is less good (probably 0.02–0.05 $\mu$).

## THE NORMAL STRUCTURE OF ARTHROPOD CUTICLE

Only a cursory summary to provide background for the following sections will be given. In current terminology the cuticle is divided into two major subdivisions distinguished by the presence and absence of the polysaccharide chitin. The outer layers of the cuticle which contain no chitin are termed "epicuticle," and the inner layers composed of a laminated chitin and protein matrix are termed "endocuticle." The epicuticle is usually distinctly double, consisting of an outer "lipid epicuticle" and an inner "protein epicuticle" (Richards and Anderson, 1942a; Dennell, 1946; Wigglesworth, 1947). Numerous further subdivisions are commonly recognizable but the above three will suffice for the purposes of the present paper.

The above three subdivisions are not necessarily all present. The cockroach has all three subdivisions in the thick cuticle covering the outside of its body and

PLATE I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also in the very thin cuticle covering its crop and its tracheae (despite the fact that these membranes are only in the range of 0.01 to 0.1 μ thick when dry). The same is true for wing membranes. Using the above terminology, the air sac and tracheae of honey bees consist only of epicuticle but have both lipid and protein subdivisions of this. The abdominal cuticle of mosquito larvae lacks a demonstrable lipid layer; it may be said to consist of a protein epicuticle plus an endocuticle. The peritrophic membrane (in the midgut) is a special case due to its peculiar origin; it contains both protein and chitin (Wigglesworth, 1939). For further analysis see papers cited in the bibliography.

Trachea are tubular invaginations of the body wall. In keeping with their origin they possess a secreted cuticle which lines the inner surface of the tubes. This cuticle is continuous with the cuticle of the external surface of the body, and the two are generally considered to be of homologous composition. These thin cuticular tubes are made more rigid by the development of supporting helical thickenings in the endocuticle. The helical thickenings are called taenidia. In large tracheae the taenidia are rather thick—too thick for adequate penetration by a 50 kV electron beam—but the membrane between the taenidia is usually quite thin (after drying ranges between 0.01 and 0.05 μ thick in species treated herein) and can be readily examined with an electron microscope (Richards and Anderson, 1942 b, c). Commonly, after removal of part of the material the taenidia decrease in density sufficiently for adequate electron penetration (Figs. 7, 17, etc.).

The intertaenidial membrane is seldom of uniform composition (Richards and

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PLATE I

Cockroach (*Periplaneta americana*), large tracheae from prothorax

FIGURE 1. Normal; intertaenidial membrane. The spots are thickenings in the endocuticle. (No. 126e) 16,000 ×.

FIGURE 2. Normal; intertaenidial membrane with edge of taenidium at lower margin; bars are braces extending from taenidium onto membrane. (No. 121d) 16,000 ×.

FIGURE 3. Normal; intertaenidial membrane with edges of taenidia at upper and lower margins. (No. 66c) 12,000 ×.

FIGURE 4. Washed with acetone, then refluxed with boiling chloroform for 5 minutes. (No. 178d) 16,000 ×.

FIGURE 5. Treated with 5 per cent KOH at 20° C. for 6 days, washed in 1 per cent HCl, then water. At left is broken, partially disintegrated epicuticle, at right only endocuticle. (No. 48c) 12,000 ×.

FIGURE 6. Treated with 20 per cent KOH at 85° C. for 4 hours, washed in 1 per cent HCl, then water. Only endocuticle of membrane. (No. 66d) 12,000 ×.

FIGURE 7. Treated with conc. KOH at 160° C. for several hours, washed in water. Taenidium vertically through center of picture, intertaenidial membrane on both sides. Resolution relatively poor. (No. 14c) 12,000 ×.

FIGURE 8. Treated with conc. KOH at 160° C. for 20 minutes, washed in water. Example with larger holes. (No. 19a) 12,000 ×.

FIGURE 9. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in alcohol. Taenidium placed vertically on right, membrane on left. (No. 198c) 12,000 ×.

FIGURE 10. Treated with 5 per cent NaOH at 20° C. for 10 days, washed in 1 per cent HCl, then water. Intertaenidial membrane only, with partially disintegrated brace extending from lower center upwards. (No. 46b) 12,000 ×.

FIGURE 11. Treated with 5 per cent NaOH at 65° C. for 6 days, washed in 1 per cent HCl, then water. Intertaenidial membrane only. (No. 32c) 12,000 ×.

FIGURE 12. Treated with 20 per cent NaOH at 85° C. for 4 hours, washed in 1 per cent HCl, then water. Intertaenidial membrane only. (No. 59e) 12,000 ×.

Korda, 1948). It usually either is studded with thickenings in the endocuticle (Figs. 1–4) or contains a reticulate meshwork of thickenings (Figs. 45–56). The structural details revealed in electron micrographs of tracheal membranes show more variability than one would like for precise quantitative work, but the changes discussed are so great in comparison to this variability that we may, as far as the present paper is concerned, treat the normal membrane structure as though constant.

## Effects of Lipid Extraction

The lipid epicuticle is not ordinarily detectable in mounts of entire membranes because it is so delicate (and seemingly so uniform) that it is lost against the stronger background of protein and chitin-protein layers. To demonstrate it convincingly with an electron microscope one has to isolate the layer (Richards and Anderson, 1942a). The figures given in the present paper show only the protein epicuticle and the endocuticle even when the lipid epicuticle is still present.

As can be seen from Figures 4 and 48, treatment with acetone and boiling chloroform produces no distinct changes in either the protein epicuticle or the endocuticle. Seemingly the effect of lipid solvents is indeed limited to removal of the superficial lipid layer, as Wigglesworth (1945) and Beament (1945) assumed.

## Effects of Chitin Purification

Extensive studies were made on the effects of chitin purification by various methods with the large tracheae from the prothorax of the cockroach as standard test material (see table). The chitin pictures obtained with this particular material were strikingly similar irrespective of the chemical method used for purification, irrespective of whether the purification was partial or what is referred to in the literature as complete, and irrespective of whether the chitin molecules were left unchanged or converted into chitosan (Figs. 5–23).

Figure 5 represents an incomplete purification with alkali. At least it is incomplete in the sense that the protein epicuticle is not completely destroyed; the epicuticle is clearly discernible in somewhat altered but recognizable condition over the left-hand part of the figure. Since it is not feasible to strip all the epicuticle off manually and test the endocuticle for proteins it is not possible to say whether the fibrous meshwork is pure chitin or still contains some protein. Ninhydrin and xanthoproteic tests on such membranes are positive but this might be due solely to the only partly disintegrated protein epicuticle. Figures 9 and 10 are other presumably incomplete chitin purifications with alkali, while Figures 6, 11 and 12 are supposedly complete chitin purifications. Figures 7 and 8 represent membranes in which the chitin has been changed into the de-acetylated form, chitosan. This picture is not significantly changed by staining with I + KI with or without a subsequent rinsing in one per cent $H_2SO_4$ (chitosan color test) or by prolonged soaking in a solution of potassium iodide. Figures 13–18 and 20 represent unknown degrees of purification with diaphanol, hydrogen peroxide, potassium permanganate, hydrochloric acid and pepsin. Figures 14 and 15 are probably pure or nearly pure chitin, the other probably only partially purified.[5] A more obscure fibrous pattern is shown by the endocuticle in Figure 23 where the treatment with hot water can scarcely have given more than a partial purification to judge from comparison with the data of Fraenkel and Rudall (1940).

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[5] We have experienced difficulty in obtaining negative ninhydrin tests on these membranes, even ones which considering the treatment should be highly purified chitin. Commonly we obtained negative xanthoproteic and Millon tests and yet a moderate positive ninhydrin test.

In all of these cases, whatever the degree of purification, the endocuticle of cockroach tracheae became primarily a fibrous meshwork (except for a delicate basal sheet to be discussed later). This is clearly not its normal state. Pictures of tracheae which have been deliberately abraded show areas where the epicuticle has been manually torn from the underlying endocuticle; in these cases the endocuticle appears as a true sheet without any discernible fibrous structure (Fig. 21). Also, so gross a fibrous structure would be discernible through the epicuticle in the control preparations (as it is in Fig. 23). Accordingly, we must conclude that the fibrous mesh is produced by the treatment.

As can be seen from the pictures given, larger fibers can usually be resolved as multiples of microfibers. The microfibers of chitin are not of constant size, but variation among them does fall within a fairly narrow range in both tracheae and other membranes. Most of the microfibers have diameters in the range of 0.01 to 0.03 $\mu$ (100–300 Å) when dry. A few less than 100 Å in diameter were estimated as approximately 75 Å. The fact that in other membranes (see below) microfibers of similar diameters may be oriented randomly suggests that there may be some real significance to these dimensions. Perhaps the microfiber diameters ($< 100 - 300$ Å) represent the range of micelle or crystallite dimensions (the *a* and *c* axes thereof) but we have no proof of this. The report by Clark and Smith (1936) of the preparation of a "micellar solution" of chitin makes more plausible the suggestion that micelles might orient into microfibers of micellar diameters on removal of other membrane components. The fact that the diameters (1 $\mu$) of the natural fibrils teased from lobster tendons by Clark and Smith are many times larger than our chemically prepared microfibers does not invalidate the above suggestion.

The chitin microfibers do not make a constant pattern except that in general they run longitudinally in the taenidia and at a right angle to this in the membrane between taenidia (Figs. 7–9, 17, 18, 20). Usually a finer reticulation is produced from gentler alkali treatment (lower concentrations and lower temperatures).[6] What seems more significant in this respect is that the pattern developed depends to some extent on the method used to remove the alkali. This effect of washing was studied in greater detail with other membranes (see below) but the generality that more destruction and coarser structure results from washing in alcohol can be seen from comparison of Figures 9 and 10. It does not seem likely, therefore, that the chitin molecules are arranged as shown in Figures 5–20 in the normal tracheal endocuticle. It seems more reasonable to assume that the chitin molecules are

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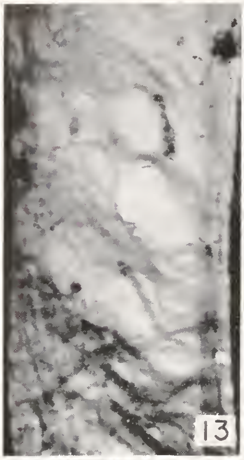
It is well known that ammonia and certain amines interfere with the ninhydrin test. Dr. W. M. Sandstrom of the Chemistry Department tells us that it would not be surprising if chitin gave a positive ninhydrin test, especially if de-acetylated. We have had to conclude that the ninhydrin test is not reliable for determining the removal of protein from chitin. Unfortunately most of our preparations were made and tested before we were aware of this, and accordingly we do not have as accurate information as we would like on the membranes used for electron microscope examination. However, subsequent tests using the same procedures indicated that the remarks in the text about purification are reasonably accurate.

[6] This is not apparent from the figures because mostly micrographs showing finer fibers and finer holes were chosen as illustrations.

## Plate II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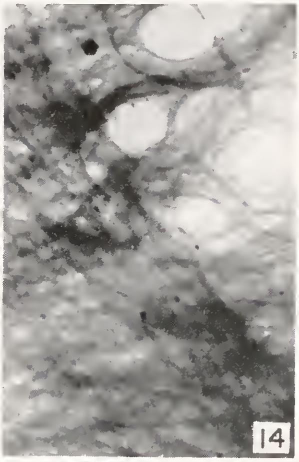
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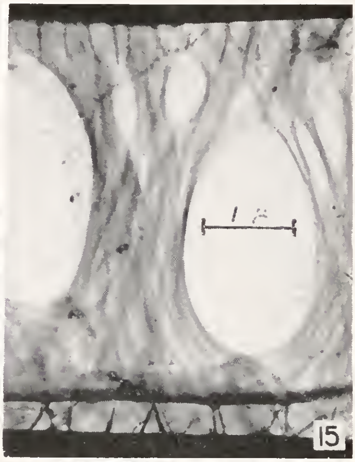

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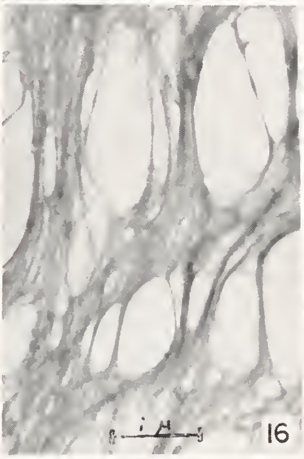

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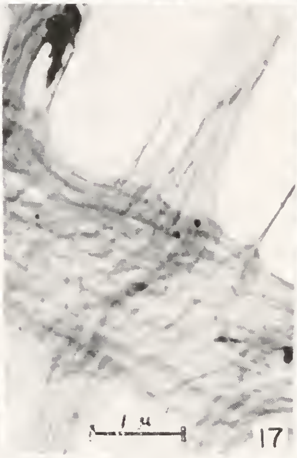

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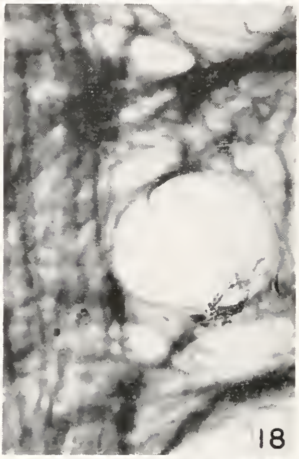
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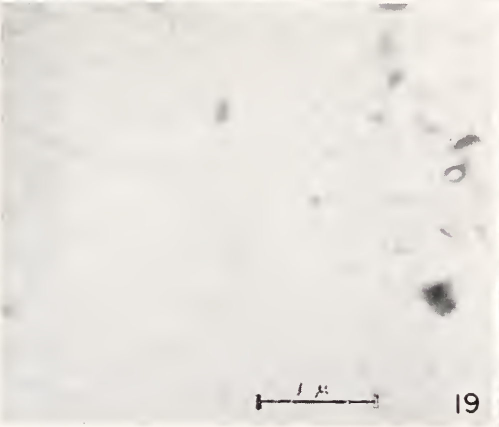

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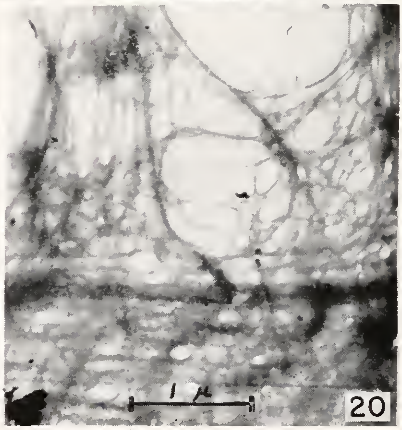

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arranged otherwise in the intact endocuticle, that they are held in place by the protein molecules, and that on removal of the protein they reorient themselves into these micro fibers which run parallel to the surface of the membrane (both before and after drying). This latter interpretation is in fair agreement with the x-ray diffraction data of Clark and Smith (1936) and Fraenkel and Rudall (1940).

Some explanation is required, however, for the consistent tendency mentioned above for the microfibers to run longitudinally in the taenidia and at a right angle to this between taenidia. The fibers in the intertaenidial membrane appear to cross the taenidia (Fig. 17), and actually a continuous fibrous membrane can be separated from the taenidia after drastic alkali treatment. It seems therefore that there is a continuous endocuticle with fibers of one orientation and attached to this the taenidia with fibers set at a right angle to those in the membrane. Both sets are parallel to the surface of the membrane, and both are distinct from the more delicate homogeneous sheet to be discussed in the next paragraph. Since chitin is an anisotropic substance it is possible to determine that there is a corresponding difference in the orientation of the chitin micelles. Using a polarized-light microscope and an appropriate compensator we have shown that when a trachea is oriented at 45° to the crossed Nicols, the taenidia are in the retarding orientation when the intertaenidial membranes are in the accelerating orientation, and vice versa. This is true for normal tracheae as well as for ones treated with alkali to purify the chitin or convert it to chitosan (amplitude of birefringence much greater after alkali treatment). It follows from this that the chitin micelles in the intertaenidial membranes are indeed at right angles to those in the taenidia.[7] This point, which is of little importance to the present discussion, will be treated further in a subsequent paper devoted to the structure of tracheal cuticles (Richards and Korda, 1948).

[7] Picken, Pryor and Swann (1947) record a mosaic of fibers for the chitin fraction of *Donacia* cocoons. While the details remain to be proven it is simple enough to rationalize such differences between tracheal and cocoon membranes on the basis of origin.

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## PLATE II

Cockroach (*Periplaneta americana*), large tracheae from prothorax

FIGURE 13. Treated with diaphanol at 25° C. for 1 week. Intertaenidial membrane; edges of taenidia at sides. (No. 50c) 12,000 ×.

FIGURE 14. Treated with diaphanol at 25° C. for 5 weeks. Taenidium and intertaenidial membrane. (No. 61a) 12,000 ×.

FIGURE 15. Treated with diaphanol at 25° C. for 5 weeks. Intertaenidial membrane with edges of taenidia at top and bottom. (No. 60a) 12,000 ×.

FIGURE 16. Treated with 30 per cent hydrogen peroxide at 20° C. for 2 days, washed in water. Intertaenidial membrane only. (No. 43b) 12,000 ×.

FIGURE 17. Treated with 0.1 per cent potassium permanganate at 20° C. for 2 days. Taenidium and intertaenidial membrane. (No. 41e) 12,000 ×.

FIGURE 18. Treated with 5 per cent HCl at 100° C. for 1 day (in sealed glass ampoule), washed in water. Taenidium on left, membrane on right. (No. 63c) 12,000 ×.

FIGURE 19. Epicuticle manually separated after treatment of trachea with 10 per cent pepsin in acid solution at 36° C. for 1 day, washed in water. Note differences in structure corresponding to taenidial and intertaenidial regions (see Fig. 20). (No. 58c) 16,000 ×.

FIGURE 20. Endocuticle manually separated after treatment of trachea with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. Taenidium at bottom, intertaenidial membrane above. (No. 58e) 16,000 ×.

In addition to the two sets of fibrous sheets treated in the preceding paragraph there is a continuous sheet in these purified tracheae. This continuous sheet is so thin and, more to the point, so homogeneous that it was overlooked by us for a long time. It is not discernable in any of the pictures reproduced here although it is present in Figures 8, 9 and some others. It is discernable in the occasional cases when it breaks after long bombardment. Once its presence was suspected it was readily demonstrated by placing small particles on it (e.g., a deposit of magnesium oxide smoke). This homogeneous sheet does not seem to be any part of the epicuticle because after separation of epicuticle from endocuticle by pepsin digestion it is found intimately associated with the chitin microfibers in the endocuticle. Likewise it does not seem possible for it to be the basement membrane of the tracheal epithelium because of alkali resistance, different appearance from known preparations of the basement membrane and intimate association with the fibrous endocuticle. It is not present in all preparations but we interpret this as probably meaning that it is commonly lost in the course of preparation. We know only three things about this membrane: its resistance to pepsin and hot alkali, its homogeneity (at least when dry) even in pictures where the resolution is to better than 50 A (0.005 $\mu$), and its intimate association with microfibers of the intertaenidial endocuticle. It is certainly a very thin membrane (probably less than 100 A thick when dry) but we do not have pictures which permit an estimation of its thickness. Perhaps it is a sheet of chitin but one would have to obtain this sheet free from the known chitinous fibers before reliance could be placed on the chitin color tests. Further work is needed on the nature of this sheet.

The protein epicuticle is destroyed by the various treatments used. However, if the treatment is slight enough to give only partial purification, a reasonably normal protein epicuticle may be peeled off the modified endocuticle. This is shown particularly well by Figure 19 (which was stripped from the preparation that gave Figure 20). This epicuticle (and also the bee air sac) never shows a fibrous structure such as the endocuticle of cockroach tracheae does. In the early stages of purification the endocuticle is more readily altered than is the protein epicuticle. This suggests that the protein is more easily removed from the chitin-protein binding than from membranes where protein chains are linked directly to one another. Otherwise stated, it would seem that, if we assume only one protein species is involved, the cuticular proteins of cockroach tracheae are less strongly bound when linked to chitin than when linked to other protein molecules.

Similar but less extensive studies were made with a number of other types of cuticle. In single tests similar results were obtained with tracheae of *Blatta orientalis* and *Galleria mellonella*, but tracheae of *Neodiprion lecontei* became only vaguely fibrous, and those of *Calandra oryzae* retained their normal appearance. The cuticle lining the crop (part of fore gut) of adult flies gave results essentially similar to those from roach tracheae.[8] On treatment with pepsin the crop membrane of a blowfly developed numerous small holes and a vague fibrous structure (Fig. 28). On treatment with hot concentrated alkali it developed a heavy fibrous mat in which the relative vagueness of the individual fibers is at least partly due to the decreased resolution occasioned by thickness of the preparation (Fig. 29). It seems that the crop membrane differs from the cockroach tracheae principally in having the chitin fibers oriented randomly and in requiring somewhat more drastic chemical treatment for purification. The crop lining of the closely related housefly gave similar results: mild alkali treatment gave relatively little change and only a very vague fibrous appearance when the alkali was removed with water (Fig. 31); removing the alkali with alcohol did not increase the fibrous appearance but, as usual (see below), resulted in a less normal appearing membrane with numerous minute holes (Fig. 32).

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[8] Considerable difficulty is encountered in getting gut membranes sufficiently clean for electron microscopy. Controls are only seldom clean enough to permit comparison with treated preparations, and then usually only clean in spots. Blurred areas on Figures 27 and 30 are interpreted as adhering debris.

The heavier membrane lining the crop of the cockroach is more resistant. Ignoring the heavy, spine-like projections and considering only the homogeneous membrane of the controls (Fig. 33), we find an irregular removal of material by hot concentrated alkali (Fig. 34). This alkali treatment was quite drastic and is commonly thought to remove all components other than chitin and also to convert all the chitin to chitosan. Yet the membrane is still intact and shows islands of greater density. Prolonged treatment with diaphonal (41 weeks) gave a different picture in which a vague, unoriented fibrous structure is apparent, with minute holes visible in the thinner parts (Fig. 35).

The peritrophic membrane of the midgut of adult flies gave pictures not encountered with any other membrane examined. This membrane has a different origin from ordinary cuticle but agrees with endocuticle in being composed of chitin and protein (Wigglesworth, 1939). Sufficiently clean controls show a seemingly homogeneous membrane (Fig. 24). Treatment with pepsin and alkali gives the same altered picture (Figs. 25–26); an intact membrane with relatively gross, denser reticulations which presumably represent reticulate thickenings (reminiscent of the reticulations seen in the normal intertaenidial membranes of many tracheae, compare Figure 55).

Wing membranes of adult mosquitoes showed the least alteration of any illustrated in this paper. Mild alkali treatment gave no detectable change (Fig. 36). Drastic alkali treatment resulted in the production of some vesicles (Fig. 37) or actual holes (Fig. 38). None of the treatments revealed any fibrous meshwork. Equally great resistance to alkali treatment was shown by centipede sensillae (Richards and Korda, 1947) and by strongly "chitinized" butterfly scales (unpublished), and by the large tracheae of the beetle, *Calandra oryzae*. These showed only a considerable decrease in density.

The cuticle of mosquito larvae has already been fractionated and studied (Richards and Anderson, 1942a). Some of these experiments were repeated especially to study the effects of various types of washing following alkali treatment. In preparing alkali-treated membranes for permeability studies Yonge (1936) washed in 95 per cent ethyl alcohol and then transferred to water for permeability tests. He assumed (erroneously) that the alkali treatment removed only the epicuticle but did not affect the endocuticle. Normal structure of mosquito larval cuticle is shown in Figure 39; it shows a fairly uniform membrane crossed by smooth parallel ridges approximately one micron apart. After an alkali treatment that should leave pure or nearly pure chitin, diverse pictures are obtained depending on the washing procedure. Washing in water results in a membrane with numerous, rather crowded, lighter spots that seem to represent vesicles in the membrane (Fig. 42); one could interpret this as representing randomly oriented vague

# Plate III

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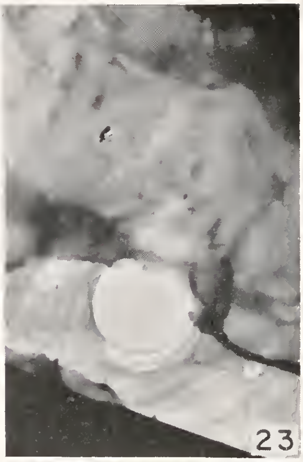
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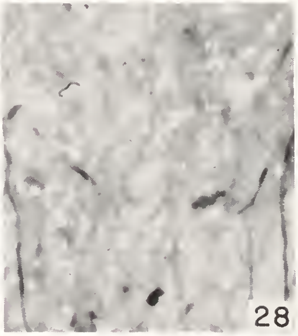
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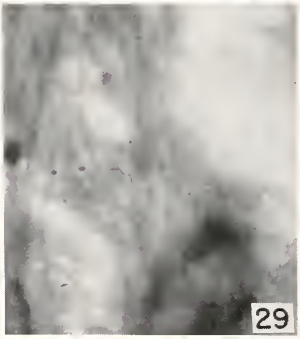
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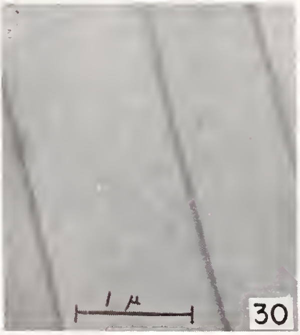

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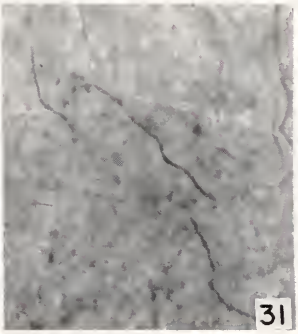
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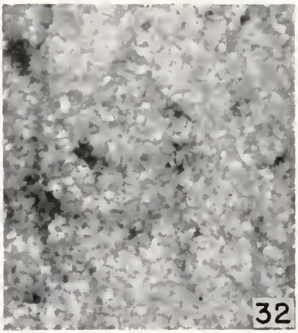
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chitin microfibers. If the treated membrane is washed in alcohol and then in water, a vague but distinct fibrous appearance is obtained (Fig. 40). If the treated membrane is washed only in alcohol many holes but no distinct fibers result (Fig. 41). If the treated membrane is washed in water first and then in alcohol a more nearly uniform membrane results with only a hazy suggestion of fibrous structure (Figs. 43–44). From this series of experiments, as well as from less extensive experiments with different washing procedures on other membranes (as noted in preceding paragraphs), it is obvious that the structure of the treated membranes is affected not only by the alkali but also by the subsequent treatment. The chitin molecules must be labile indeed for a water treatment to return a membrane such as that shown in Figure 41 to the condition of that shown in Figure 40. It does not seem desirable to attempt interpreting the diverse set of pictures shown in Figures 40–44 other than to say that clearly no one of them is "normal," that no one would have any biological significance if used in a permeability experiment and that washing alkali out with alcohol seems to cause more deviation from normal membrane structure than washing with water does.

## Effects of Extraction with Water and Aqueous Solvents

Fraenkel and Rudall (1940, 1947) and others have shown that some of the protein of insect cuticles is extractable with hot water. There is an inverse relationship to sclerotization, i.e., soft, non-sclerotized cuticles lose much of their protein on extraction with hot water, while hard, sclerotized cuticles lose relatively little.

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### Plate III

All figures reproduced at 16,000 ×

Figure 21. Cockroach trachea. A normal trachea manually abraded. The epicuticle has been torn and peeled away from a small area leaving the intact endocuticle exposed. (No. 384c)

Figure 22. Cockroach trachea. Treated with distilled water at 100° C. for one week. (No. 336e)

Figure 23. Cockroach trachea. Treated with distilled water at 100° C. for 2 weeks. Area of approximately maximum effect. (No. 340d)

Figure 24. Blowfly (*Lucilia illustris*), peritrophic membrane. Normal, washed in water but probably not entirely clean. (No. 89e)

Figure 25. Blowfly, peritrophic membrane. Treated with 40 per cent KOH at 100° C. for 10 minutes, washed in water. (No. 87b)

Figure 26. Blowfly, peritrophic membrane. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 78b)

Figure 27. Blowfly, crop lining. Normal, washed in water but not entirely clean. (No. 79b)

Figure 28. Blowfly, crop lining. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 80c)

Figure 29. Blowfly, crop lining. Treated with conc. KOH at 160° C. for 15 minutes, washed in water. (No. 79d)

Figure 30. Housefly (*Musca domestica*), crop lining. Normal, washed in water but speckles may be debris. (No. 193c)

Figure 31. Housefly, crop lining. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in water only. (No. 194a)

Figure 32. Housefly, crop lining. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in 95 per cent ethyl alcohol. (No. 195a)

Since isolated cuticles are sometimes used in permeability studies, in our laboratory and elsewhere, it seemed desirable to see what we could learn from electron micrographs of membranes extracted with water and salt solutions at various temperatures.

Cockroach trachea soaked in distilled water at 25° C. for periods up to two weeks appeared normal. Ones soaked at 60° C. for one and two week periods seemed to be somewhat affected but the differences from control preparations were not well marked. Ones soaked at 100° C. showed a clear loss of substance, development of a network type endocuticle with some fibrillar structure, and a considerable tendency for the epicuticle to break (Figs. 22–23). Cockroach tracheae exhibit so much variability that they are useful only when the effects are striking (as in chitin purifications) and accordingly it is not safe to draw conclusions from the negative results of extraction with water at low temperatures.

The air-sacs of honey bees gave more clearly defined changes and so were studied rather intensively.[9] As far as can be ascertained from structure visible in the electron micrographs, soaking these membranes in distilled water at 25° and 30° C. for periods up to a week produces no change. At the higher temperatures which Fraenkel & Rudall used for extracting proteins from cuticles, the effects are striking. Air-sacs treated in distilled water at 65° C. for two days show obvious swelling of the ridges accompanied by decrease in sharpness and density (Fig. 49). Electron micrographs of air sacs treated with hot water do not always give this particular type of picture but do always contrast with control preparations. Another type of picture is shown by Figure 50, of a specimen soaked at 65° C. for a week; in this case the removal of material and the change has not been uniform over the preparation—as can be seen by comparison of right and left halves of this picture. Treatment with distilled water at 100° C. gives still greater change. After one day there is a general fuzziness suggesting removal of material from the ridges (Fig. 51). After eight days the membrane is much more delicate, the ridges and reticulations have mostly disappeared and the membrane between ridges is less dense and therefore thinner (Fig. 52). After a month soaking at 100° only a very delicate, but still continuous, membrane remains (Fig. 53).[10] The delicate membrane remaining in Figure 53 does not show properties of the lipid epicuticle, i.e., it does not disintegrate under electron bombardment; accordingly it may be presumed to represent one of the protein layers. These hot water extractions of air sacs show, as one might have predicted from the work of Beament (1946) and Wigglesworth (1947), that the protein is more readily removed from some portions than from others; seemingly protein is removed most readily from the thickenings and less readily from some of the continuous layers than from others. Whether there are as many discrete layers in the bee air sac as in the thicker epicuticles studied by Beament and Wigglesworth is not known but analysis of these extraction pictures suggests that more than one protein layer is present. This is also suggested, but not proven, by the membranes remaining after pepsin digestion (Figs. 54–55).

The resolution shown in electron micrographs of bee air sacs is excellent.

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[9] One should remember that the air-sacs of honey bees are consistently negative to chitin tests.

[10] For focusing electron micrographs some contrast is needed. With such homogeneous membranes this is most readily accomplished, as was done here, by finding an area where minute particles of debris are present.

Sharp discontinuities of the diameter of 100 Å should be clearly visible, and in the best pictures sharply contrasting discontinuities (e.g., holes) of half this size should be detectable. However, since we are dealing with a multiple membrane where effects in one layer may be partially hidden by an overlying layer we cannot claim to have proven the absence of effects of this magnitude. To be on the conservative side we will only say that there seem to be no changes down to the range of several hundred Ångstrom units. One could therefore claim that there is good evidence that these air sacs could be used as isolated membranes for permeability studies at room temperatures. At high temperatures they are altered but at room temperatures even rather lengthy treatments give either only exceedingly slight alterations (beyond detection by the present electron micrographs) or else slight effects which are completely masked by drying.

Soaking honey bee air sacs in physiological saline solutions for several days at 25° C. likewise seems to cause no detectable effects (Fig. 48). The same may be said for dilute solutions of some single salts (e.g., KCl). However, it seems likely that strongly hypertonic salt solutions do affect these membranes deleteriously. The effects of hypertonic saline solutions are only slight; an adequately extensive analysis of them has not been completed. We can safely record, however, that strong aqueous solutions of agents commonly used to facilitate protein extraction can affect the structure seen in electron micrographs of bee air sacs (e.g., 6M urea, 5 per cent sodium salicylate, detergents). A number of detergents (anionic, cationic and non-ionic) have been tested in a preliminary manner. Different detergents do not give the same degree of effect but even in dilute solutions they show a considerable disruptive effect (Fig. 56). This fact is of some interest because Wigglesworth (1945), in studying cuticle permeability, compares the effect of removing the lipid epicuticle by the use of lipid solvents with the presumed disruption of the lipid epicuticle by the use of detergents. Clearly this comparison is not a simple one because we can demonstrate no effect for lipid extraction other than the removal of the lipid epicuticle whereas detergents disrupt both the lipid epicuticle and at least some of the protein components of the underlying membranes.

The wings of mosquitoes remain intact after washing in distilled water at various temperatures for various periods. Illustrations are not given. The appearance of normal and control membranes is as shown in Figure 36. After soaking in distilled water, even at room temperatures, the membranes become less dense; this is shown particularly by an increase in contrast between the general membrane and the small darker (denser) areas.[11] Such increased contrast and decreased density is more marked with wing membranes soaked in hot water. It seems that some of the diffuse material is removed by water. Interpretation of the decreased density shown by electron micrographs of wing membranes is difficult. Resolution in these thick, multilayered membranes is relatively poor even in the best pictures, and accordingly any structural alteration would have to be relatively gross to be detected (at least several hundred Ångstrom units). Furthermore, wings consist of two membranes separated by the remnants of dead epithelial cells; it is conceivable that the material removed by water is largely this cellular debris. As far as can be determined from our pictures the wing membrane maintains its integrity. What

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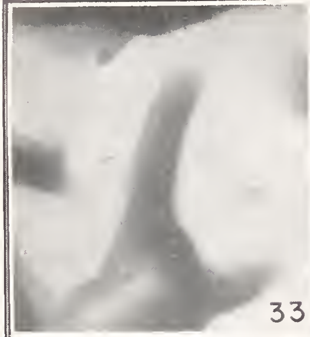
[11] The identity of these small dark areas has not been definitely established but it seems likely that they represent thickenings in the endocuticle comparable to those in cockroach tracheae.

## Plate IV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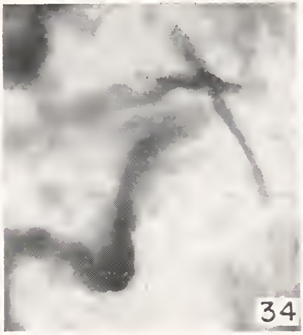
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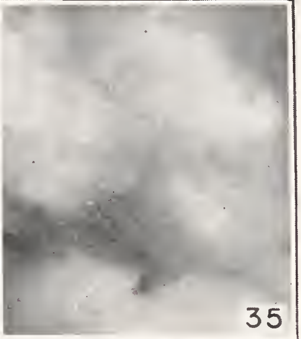
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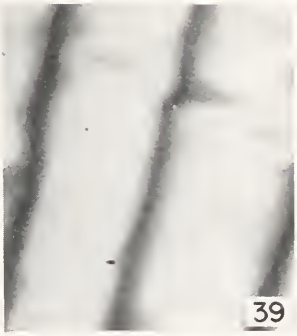
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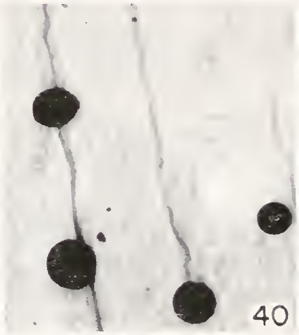
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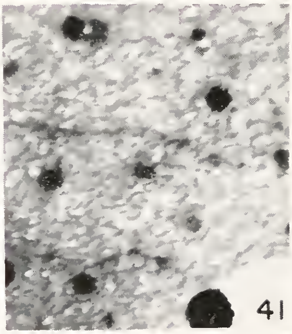
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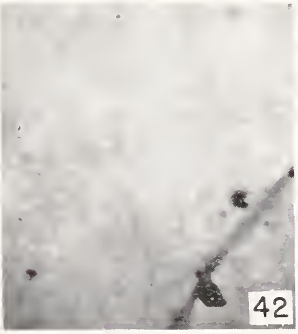
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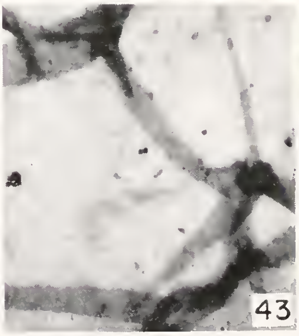
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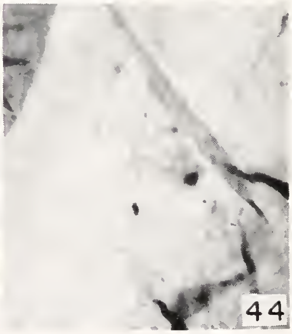
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can be stated definitely is that these wing membranes do not become altered in water in a manner comparable to or to the extent that cockroach and bee tracheae do.

The soft, non-sclerotized abdominal cuticle of mosquito larvae was tested as an example representing approximately the other extreme from wing membranes. Normal structure is as shown in Figure 39. Soaking isolated, cleaned cuticles for one day or one week in distilled water at 20° C. produced no positively indentifiable alterations but again it should be stressed that resolution in such thick membranes is not very good (probable order of several hundred Ångstrom units). Soaking isolated, cleaned cuticles for one day or one week in distilled water at 65° C. produced marked changes; the results were not entirely constant but were all in the intermediate range between patterns shown by Figures 40 and 42. Treating living larvae with water at 65° C. (which is lethal) for a minute and then removing the cuticle and brushing it clean gave preparations indistinguishable from the controls, but treating such intact larvae with water at 65° C. for an hour produced a fibrous appearance comparable to that obtained by soaking isolated cuticles in water at this temperature. Soaking isolated cuticles for one day or one week in distilled water at 95° C. gave pictures more nearly like Figure 44. No interpretation is made for these results beyond saying that there are obvious structural changes following treating or soaking at 65° and 95° C. but that there are no clearly demonstrable changes following soaking at 20° C.

The above results may be compared with the data published by Fraenkel and Rudall (1940, 1947). Soaking in water at room temperatures causes very little

PLATE IV

All figures reproduced at 16,000 ×

Figure 33. Cockroach, crop lining. Normal, washed in water. Dark structures are heavy projections. (No. 81b)

Figure 34. Cockroach, crop lining. Treated with conc. KOH at 160° C. for 15 minutes washed in water. (No. 81c)

Figure 35. Cockroach, crop lining. Treated with diaphanol at 25° C. for 41 weeks. (No. 251c)

Figure 36. Mosquito (*Aedes aegypti*), wing. Treated with 5 per cent KOH at 100° C. for one day. Not significantly different in appearance from control. The scattered darker areas appear consistently on normal and water-washed mosquito wing membranes but their identity has not yet been established. (No. 90d)

Figure 37. Mosquito, wing. Treated with conc. KOH at 160° C. for 15 minutes, washed in water. (No. 91b)

Figure 38. Mosquito, wing. Treated with 20 per cent KOH at 100° C. for one day, washed in water. (No. 91a)

Figure 39. Mosquito larva (*Aedes aegypti*), abdominal cuticle. Normal, washed in water. (No. 372e)

Figure 40. Mosquito larva, abdominal cuticle. Treated with 20 per cent KOH at 65° C. for 48 hours, washed in 95 per cent ethyl alcohol, then in water. (No. 368e)

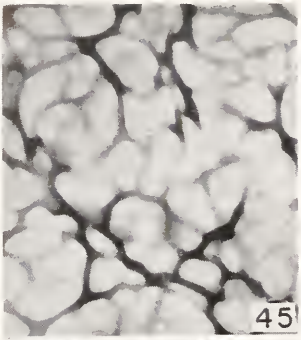
Figure 41. Mosquito larva, abdominal cuticle. Same treatment but washed only in 95 per cent ethyl alcohol. (No. 368d)

Figure 42. Mosquito larva, abdominal cuticle. Same treatment but washed only in water. (No. 367b)

Figure 43. Mosquito larva, abdominal cuticle. Same treatment but washed in water, then in 95 per cent ethyl alcohol. (No. 367d)

Figure 44. Mosquito larva, abdominal cuticle. Same treatment but washed in water, then in 95 per cent ethyl alcohol, then in water. (No. 368b)

## PLATE V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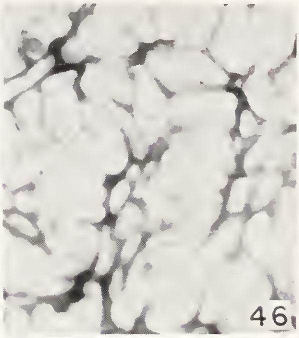
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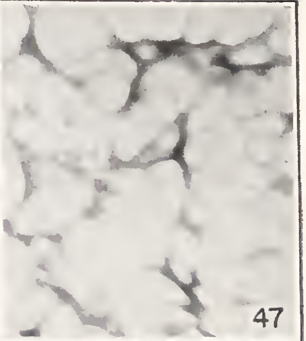
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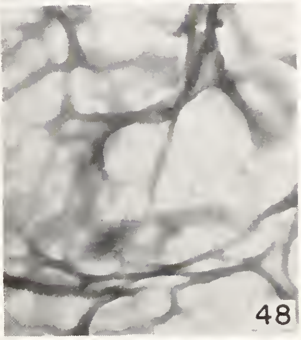
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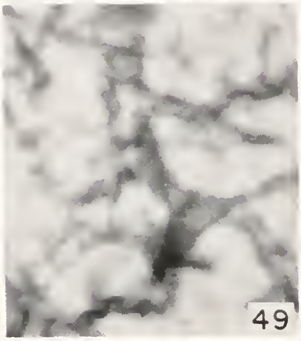
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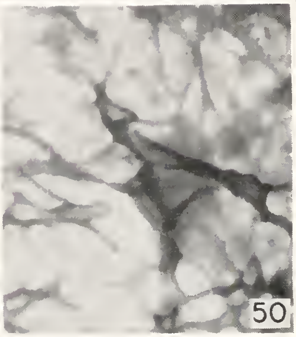
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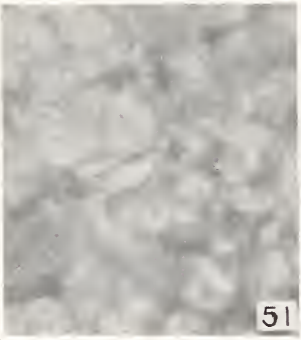
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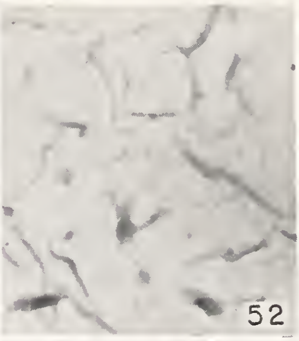
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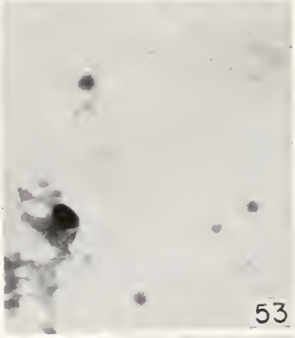
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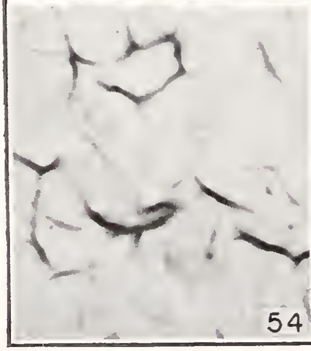
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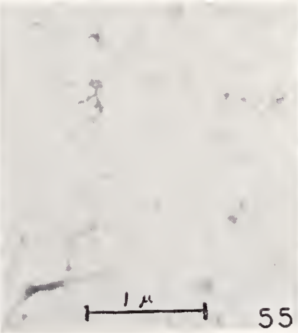

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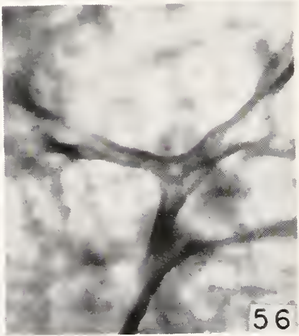
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loss of weight and no detectable change in electron micrographs. Soaking in water at high temperatures causes considerable loss in weight (at least largely removal of protein) and more or less extensive alteration of structure shown in electron micrographs.

All of the above examples are free from gross discontinuities (e.g., pore canals, gland ducts). The electron micrographs show that under favorable conditions changes that would invalidate permeability data do not occur at least down to the range of a few hundred Ångstrom units. However, most molecules of interest in studies of cuticle permeability are much smaller than this. Electron microscope studies show that numerous procedures introduce alterations that invalidate the use of such treatments of isolated cuticle but resolution in electron micrographs of cuticle is not fine enough to permit saying that no significant alterations occur after those treatments for which we could detect no changes. Other tests should also be used (e.g., change in membrane potential on soaking, etc.) and even with all possible checks final validation of the precise measurements possible with isolated cuticle will have to depend both on absence of demonstrable change and integration of the results with data from intact animals.

## Discussion

The purification of chitin in membranes usually leads to extensive structural alterations demonstrable by electron microscopy. It follows that such purified chitin membranes can have no real biological significance in permeability studies. It also follows that we cannot continue to hold the old view that arthropod cuticle consists of a framework of chitin in which other components are merely embedded (see Fraenkel and Rudall, 1940, 1947; Richards, 1947 a, b).

Of more interest is the unexpected degree of dissimilarity of chitin patterns given by different types of cuticular membranes. The pattern obtained in a specific case is primarily correlated to the type of cuticle used rather than to the chemical treatment, and results of chitin purifications are reproducible with reasonable quali-

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### Plate V

Honey bee (*Apis mellifica*), abdominal air sac. All figures reproduced at 16,000 ×

Figure 45. Normal, washed quickly in distilled water. (No. 229a)

Figure 46. Soaked in distilled water at 25° C. for 2 days. (No. 238e)

Figure 47. Soaked in isotonic saline at 25° C. for 2 days. (No. 253c)

Figure 48. Refluxed with boiling chloroform for 2 hours. (No. 228e)

Figure 49. Soaked in distilled water at 65° C. for 2 days. (No. 222a)

Figure 50. Soaked in distilled water at 65° C. for one week. Note differences between right and left halves indicative of more extraction from some areas than others. (No. 222e)

Figure 51. Soaked in distilled water at 100° C. for one day. (No. 323e)

Figure 52. Soaked in distilled water at 100° C. for 8 days. (No. 345b)

Figure 53. Soaked in distilled water at 100° C. for one month. Membrane so delicate that detectable in electron micrographs principally by small particle of debris on it. (No. 370d)

Figure 54. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 225c)

Figure 55. Refluxed with boiling chloroform for 2 hours, washed through acetone to water, and then treated with 10 per cent pepsin in acid solution at 36° C. for one day. (No. 228b)

Figure 56. Soaked in 0.02 per cent "Aerosol OT" (di octyl sodium sulfo succinate) in distilled water at 25° C. for 24 hours, washed in water. (No. 288a)

tative precision. Even though one can alter the pattern considerably after the purification treatment (alcohol versus water wash), we have not been able to obtain a single type of pattern from all types of cuticle by variations in technique. Depending on the type of membrane used, one obtains either a fibrous mat of greater or lesser degree of orientation, a vesicular membrane which is partly a fibrous feltwork, a reticulate membrane without distinct fibers or a seemingly homogeneous membrane with some small holes. If the continuous sheet in cockroach tracheae is found to be chitin we would have to add to this list a membrane homogeneous to less than 50 Å, at least when dry. These differences are not functions of membrane thickness and not functions of per cent de-acetylation of chitin to chitosan. Suggestions for an explanation of these diverse results would include: (a) a function of the relative percentage of chitin and protein in the membrane; (b) a function of degree of sclerotization; (c) a function of alkali-insoluble impurities; and (d) a function of different degrees of chitin polymerization. All four suggestions imply that chitin molecules vary either in size or in intermolecular bindings, and therefore they overlap and become aspects of one another.

The possibility of these diverse patterns being caused simply by variations in percentage of chitin present seems to us unlikely. There is really no convincing evidence for this statement. One can readily imagine variation in chitin percentage affecting the number of fibers per unit area, but we do not see how chitin percentage *per se* could account for fibrous networks versus homogeneous membranes, especially when the microfiber diameters (when found) are similar in different membranes, unless we also assume differences in cross linkages (which automatically moves the question to the fourth point).

Actually, the determination of the percentage of chitin present in any membrane is difficult, and we have only a crude idea of the accuracy of available methods (Campbell, 1929; Fraenkel and Rudall, 1947; Richards, 1947b). For most of the membranes studied in the present work it is not feasible to attempt such a determination. In current literature there are two opposed viewpoints on chitin-protein percentages in arthropod cuticle. Fraenkel and Rudall (1947) postulated a basically constant chitin percentage based on a hypothetical model of alternating monolayers of chitin and protein and assume that all deviations are secondary. Richards (1947) postulated a basically variable percentage of chitin based on the fact that all membranes have a high percentage of protein whereas the chitin percentage may range from 0 to 60 per cent. It seems to us difficult to reconcile our results with the alternating monolayer concept of Fraenkel and Rudall. The narrow range of microfiber diameters is readily interpretable on the idea of there being macromolecular chitin micelles which form mixed crystals with the protein molecules which have nearly identical lattice unit dimensions, but it would be surprising if monomolecular layers of chitin would reorient into macromolecular microfibers of such constant dimensions following removal of protein. Macrolayers in the cuticle are evident (e.g., interference color phenomena, alternating laminae in electron micrographs) but monolayers or even single micellar layers are not. At present all ideas are only working hypotheses.

The second possibility, variation in degree of sclerotization, is difficult to analyze. By definition, sclerotization is the formation of sclerites, i.e., hardened areas in the cuticle. Campbell (1929) was the first to show that this hardening is not due to chitin *per se*. Pryor (1940) elucidated part of the picture by demonstrating the tanning of cuticular proteins by oxidized orthodihydroxyphenols with concurrent hardening. If this were the whole story of sclerotization the process should not affect the structure of purified chitin. But x-ray and other data lead to the assumption of chitin-protein bindings, the nature of which is not known. Our work shows that purification of chitin has less destructive effect on sclerotized membranes than on non-sclerotized membranes despite the fact that the sclerotized membranes contain lower percentages of chitin (Fraenkel and Rudall, 1947). It seems that as a result of sclerrotization the chitin molecule linkages are so changed that a different pattern is obtained following so-called purification of the chitin. Again we are led to the idea of variations in chitin cross-linkages to account for our diverse pictures even if the diversity is found to be a by-product of sclerotization.

The third suggestion, variable alkali-insoluble impurities, is at present only a conjecture. One of the great difficulties of work on cuticle is that we have no means of determining the degree of purification of chitin. It is quite possible that no one has ever seen pure chitin (note the low nitrogen value consistently obtained by Fraenkel and Rudall, 1947). Solution in acid leads to rapid hydrolysis of ether linkages (Clark and Smith, 1936) and it is not certain that solutions in lithium thiocyanate are free from impurities. Until we have conclusive analyses there is little that can be done about this uncertainty.

The fourth suggestion, various degrees of polymerization of chitin molecules, seems to fit our diverse pictures best but it cannot be claimed that our data prove this to be true. Chitin, unlike cellulose, is not known to occur naturally in anything approaching the pure state. It is customarily assumed that there must be chitin cross-linkages to or through other compounds but the possibility of various degrees of cross-linkages between chitin molecules seems not to have been suggested—no doubt largely because nothing definite is known about it. When microfibers are obtained they show a rather narrow range of diameters irrespective of whether they parallel one another or run randomly. This suggests there is some real significance to the values (micelle diameters ?), but the absence of such microfibers in certain purified membranes would seem to necessitate a different type or degree of cross-linkages between chitin chains or chitin micelles.

To account for our diverse electron microscope pictures there must be different degrees or kinds of reorientation of chitin molecules following removal of other membrane components. Unfortunately we can do no more than speculate about this at present. Conceivably such results might be a function of chitin chain lengths (if these vary like cellulose chains), or of stable cross-linkages present before purification, or of different spatial patterns in the cuticle tending to different degrees of chain cross-linkages (hydrogen bonds ?) following purification, or of variable amounts of stabilizing impurities if such exist. Apparently these linkages would not involve the acetyl group since purified chitin and chitosan membranes give similar electron micrographs. Obviously we need to know more about chitin chemistry on the polymer level.

## SUMMARY

1. Chitin purifications alter the structure of cuticular membranes of insects more or less extensively.

2. Our data agree with the x-ray diffraction studies of Fraenkel and Rudall in showing:

(a) The cuticle cannot be viewed as a rigid chitin framework in the interstices of which other components are deposited.

(b) Protein extraction methods have no value for localization of membrane components.

(c) Purified chitinous membranes have no significance for studies on the permeability of arthropod cuticle.

3. Several but not all types of membranes after purification yielded microfibers of chitin which after drying have diameters of $< 100$ to 300 Å ($< 0.01$ to 0.03 $\mu$). It is suggested that these values may represent chitin micelle diameters.

4. Unexpected diversity in chitin patterns was obtained. Possible interpretations are discussed, and it is suggested that chitin cross-linkages vary considerably from one type of membrane to another type.

5. Chloroform and acetone remove the lipid epicuticle without affecting the structure of the underlying protein epicuticle and endocuticle. Detergents disrupt both the lipid epicuticle and underlying protein layers.

6. The effects of water and salt solutions on the structure of isolated cuticular membranes depend on temperature, the salt used and its concentration and the type of membrane. There is good correlation between weight losses recorded at various temperatures by Fraenkel and Rudall and structural changes found in our work. It is concluded that isolated cuticular membranes that are free from gross discontinuities can be used to a certain extent in permeability studies employing methods of physical chemistry but only when parallel tests show that the particular membranes remain reasonably near their original structure and composition.

## LITERATURE CITED

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# SEASONAL AND ANNUAL VARIATIONS IN THE ATTACHMENT AND SURVIVAL OF BARNACLE CYPRIDS[1]

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Two factors governing the development of a barnacle population have been investigated: namely, the number of barnacle cyprids attaching daily and the relationship of the daily cyprid accumulation to the number of adult barnacles attaching per month in Biscayne Bay, Miami Beach, Florida. On surfaces exposed for one month at this location, adult barnacles were invariably the dominant organism. No competition by other forms developed in this time interval to limit significantly the attachment of barnacles or their growth. This study was therefore concerned with numbers of cyprids settling daily, survival of cyprids, seasonal growth rates and space limitations, as factors affecting the size of the month-old barnacle population.

The survival of barnacles following attachment has been studied principally by Moore (1934), Hatton (1938) and Moore and Kitching (1939) with respect to the attachment of *Balanus balanoides* and *Chthamalus stellatus* to cleaned rock surfaces in the intertidal zone. These investigations were interested mainly in the effect of tidal level, exposure to surf and to sun, and interspecific competition on the growth and mortality of intertidal barnacles. Their findings are not directly applicable to barnacles attached to continuously submerged surfaces since they emphasize the effect of tidal exposure. The investigations of van Breemen (1933) on the survival of barnacles attached to continuously immersed surfaces considered the ultimate age of specific species.

## METHODS

To ascertain the numbers of barnacle cyprids attaching daily, a collecting surface of smooth, black glass was hung in the waters of Biscayne Bay at the Beach Boat Slips, Miami Beach, Florida, just below the low-tide mark. The cyprids which attached to one side of the glass (500 sq. cm.) during each 24-hour period were counted daily between 8:00 A.M. and 8:15 A.M. The surface was wiped clean after each counting. Temperature and density of the surface water were also determined at the same time. From these data salinity was calculated. The investigation was carried out over a period of 38 months, except for short interruptions in the summers of 1943, 1944 and 1945.

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During the same period, monthly fouling attachments in Biscayne Bay were also reported. These fouling collections were made on clear glass panels, backed with a dark red paint, of the same area as the cyprid collecting surface. The dark red backing provided a substratum color which was comparable to the black glass (Visscher and Luce, 1928). A total of 74 monthly collections were made, panels being immersed every 15 days for 30 days of exposure. From the monthly attachment of assorted fouling forms the barnacle count was extracted for comparison with the sum of the daily cyprid attachment for the same month. *Balanus improvisus* was the dominant adult barnacle on the monthly collectors and presumably was responsible for most of the cyprids (Weiss, 1948).

## Results

The daily records of cyprid attachment, water temperature and salinity are presented in Figures 1, 2 and 3, respectively. Three-day moving averages were employed in plotting these data, for the purpose of minimizing random fluctua-

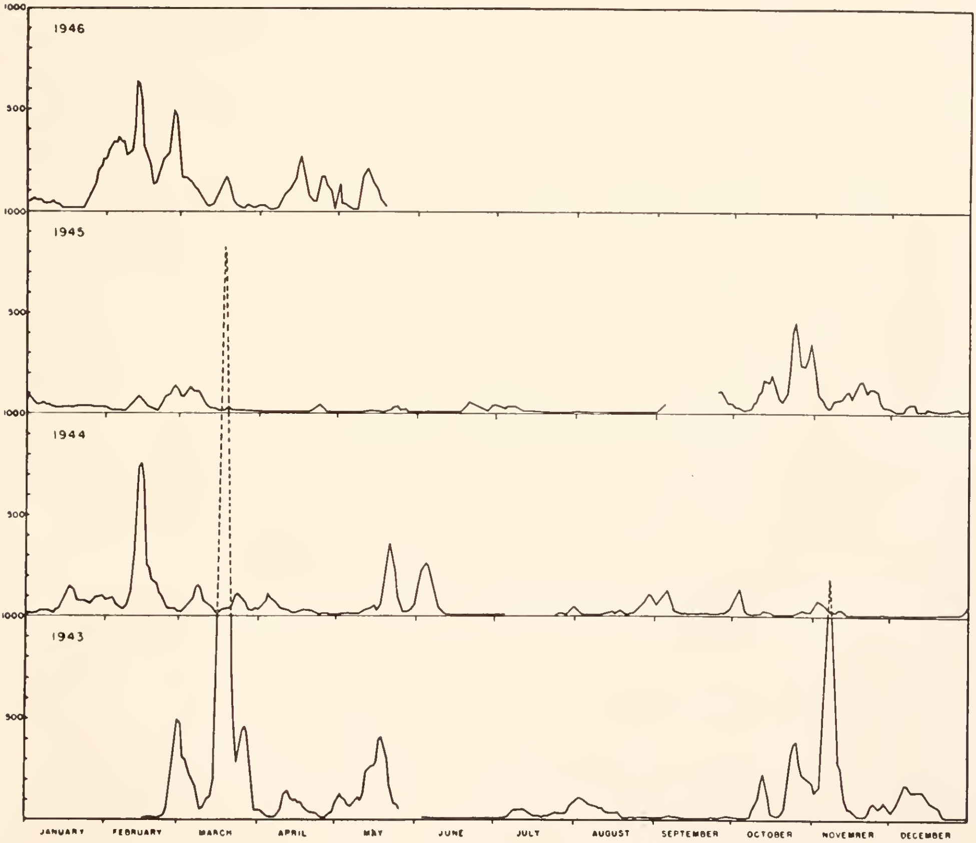

FIGURE 1. The daily accumulation of barnacle cyprids on black glass (500 sq. cm.) at the Beach Boat Slips, Miami Beach, Florida, February 1943–May 1946, plotted as a three-day moving average.

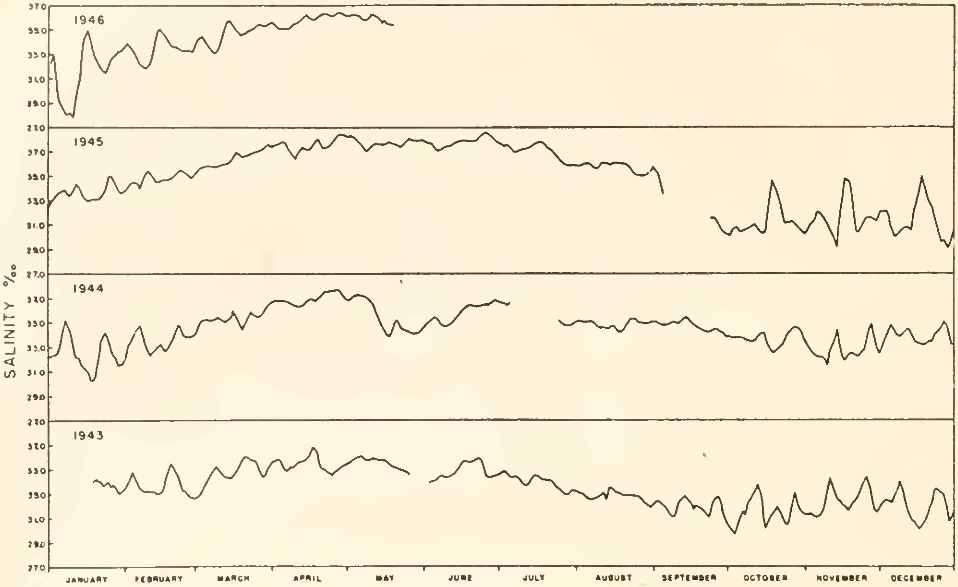

FIGURE 2. The daily salinity record of Biscayne Bay made at the Beach Boat Slips, Miami Beach, Florida, plotted as a three-day moving average.

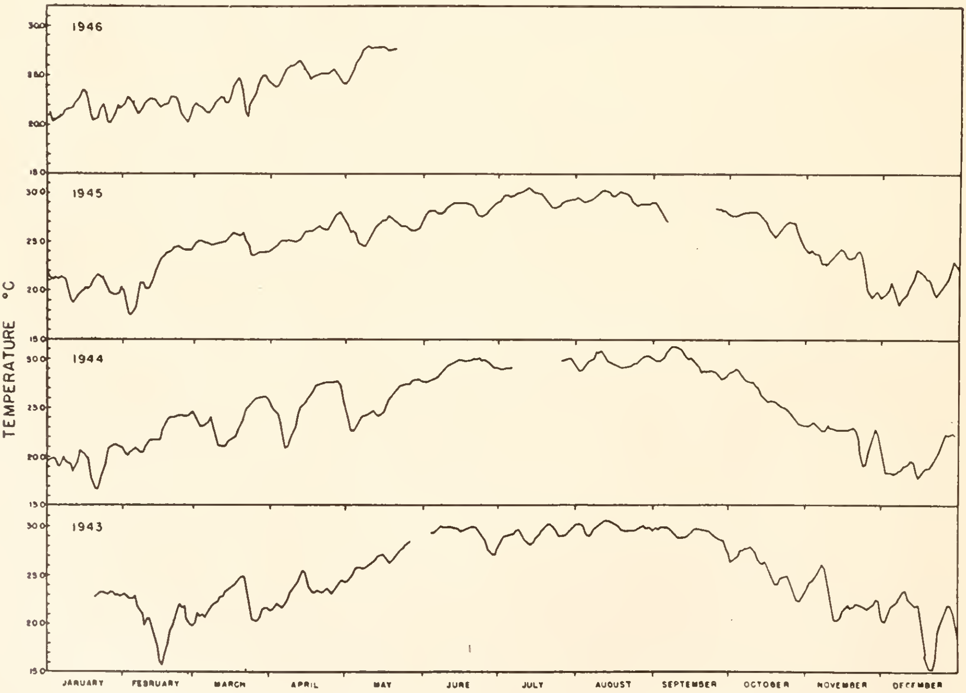

FIGURE 3. The daily temperature record of Biscayne Bay made at the Beach Boat Slips, Miami Beach, Florida, plotted as a three-day moving average.

tions. Both the temperature and salinity of Biscayne Bay showed seasonal variation. The temperature fluctuated each year between approximately 16° C. and 30° C. The salinity showed an annual range from approximately 28.00 $^0/_{00}$ to 37.00 $^0/_{00}$, the peak coinciding with the end of the dry season. This period occurred each year between April and June. With the onset of the summer rains, the bay salinity declined, reaching its minimum in the period from October to January. During this period, and to a lesser degree in the month preceding and following, the salinities showed marked short-period fluctuations, which were indicative of the stage of tide at which the water sample was taken. A sample taken at high tide was always more saline than one sampled at low tide. This fluctuation of salinity was most pronounced during the rainy season, when the surrounding land areas were well soaked and the fresh water run-off from the land diluted the bay water. Slight tidal variations in salinity were evident even in the period of maximum salinity, but the range was not as great. Temperature fluctuations paralleling the tidal cycle were also evident due to the more rapid warming and cooling of the comparatively isolated bay water.

Barnacle cyprids settled on every day but three during the 38 months of observation. The numbers attaching daily varied from as low as 1 or 2 to a maximum of over 2800 per 500 sq. cm. In general, the settling of cyprids was characterized by peaks in attachment at relatively frequent intervals. Nevertheless, there was no apparent relationship between the sizes of successive peaks and the interval between the peaks, which ranged in most cases from 7 days to a month.

Several peaks of cyprid attachment occurred in the early spring and late fall of each year, but these peaks showed considerable variation from year to year in both magnitude and exact time of occurrence. The period from June to September was characterized by low daily cyprid attachments and a few small peaks, if any. However, in 1945 this period of reduced daily attachment extended from the middle of March to September. The spring peaks of attachment were generally greater than those of the fall; but in 1945 this pattern was reversed, the fall peaks being greater. The spring peaks in 1946, although large, were not as great as in 1943 and tended to extend over a longer period.

For the purpose of ascertaining the mechanism controlling the periodic rises in cyprid attachment, these fluctuations were analyzed with respect to changes in the temperature and salinity of the bay water. Since the barnacle cyprid follows after a series of six nauplii stages, fluctuations of its attachment were also examined with respect to changes in the marine environment taking place at the time of release of the nauplii into the plankton. A search of the literature failed to reveal the duration of the free-swimming period for *Balanus improvisus*. However, of the species that were studied (Table I), the free-swimming periods ranged from 7 days to 3½ weeks.[3]

Assuming intervals of one, two or three weeks between spawning and settling, no satisfactory correlations were found between changes in temperature and salinity and the periodic peaks in cyprid settling. The only relationship evident was between the seasonal increases in numbers settling and the temperature. The rises

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[3] The period of time that the cyprid spends in the plankton prior to settling was noted quantitatively by Willemoes-Suhm (1876), who studied the development of *Lepas fascicularis*. In addition to observing six nauplii stages, he noted that the "cyprid evidently attaches itself on the very first occasion."

TABLE I

*Time for development through nauplii stages to cyprid*

| Barnacle species | Number of nauplii stages | Time for development to cyprid |
|---|---|---|
| *Balanus crenatus* Bruguiere [1] | 6 | 7–10 days |
| *Balanus crenatus* Bruguiere [2] | 8 | 13–14 days |
| *Balanus eburneus* [3] | 6 | 7–10 days |
| *Balanus perforatus* Bruguiere [4] | 6 | 21 days |
| *Chthamalus stellatus* [5] | 6 | 18–20 days |
| *Verruca stroemia* [5] | 6 | 23–27 days |
| *Balanus balanoides* [5] | 6 | 3–3½ weeks |

[1] Bohart (1929).
[2] Herz (1933).
[3] Grave (1933).
[4] Lochhead (1936).
[5] Bassindale (1936).

in cyprid numbers in the spring and autumn occurred between the periods of minimum and maximum temperatures. This relationship suggested that *Balanus improvisus* breeds in a temperature range of approximately 18° C. to 27° C. The temperature above which breeding diminished greatly or ceased was more clearly defined than the minimum temperature limit. The waters of Biscayne Bay exceeded the upper temperature breeding limit for several months each year, but rarely and only for short periods did the temperature drop below the lower breeding limit.

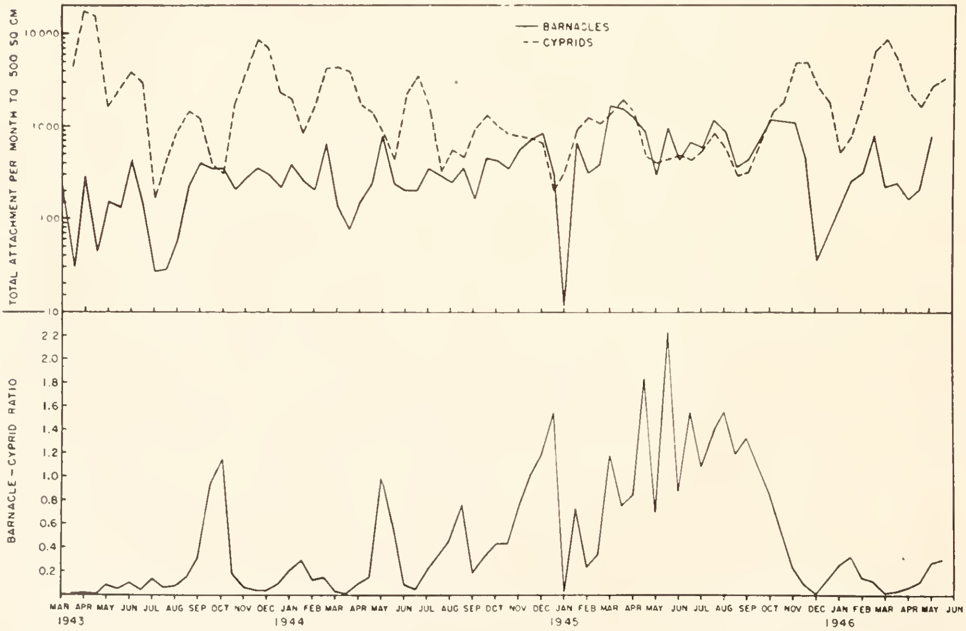

FIGURE 4. The monthly totals of daily cyprid settling and the monthly barnacle attachments on glass surfaces (500 sq. cm.), upper half. The ratio of barnacles to cyprids attached in each monthly period, lower half.

The numbers of barnacles found on the monthly collectors showed considerable variation, as indicated in Figure 4. For the entire period of observation the monthly barnacle collections dropped below 100 only eight times and went above 1000 only six times. The extreme numbers of barnacles collected in one month were 10 in December 1944 and 1615 in February 1945. However, extreme fluctuations in numbers of barnacles did not usually occur in two successive collections but followed a period in which a trend of rising or falling numbers was evident. Although not as marked, a rising trend in the monthly collections of barnacles during the period of 1943–1945 was observed, reaching a peak simultaneously with the low point of the monthly totals of cyprids.

Of the varying numbers of cyprids attaching daily it was assumed that all or some portion metamorphosed and grew to adult size. For comparison with the monthly barnacle collections the daily cyprid attachments were totaled every 15 days for the preceding 30-day period. The monthly cyprid totals, in general, showed a greater fluctuation than the monthly barnacle collections, as indicated in Figure 4. During the 38 months of observation, the numbers of cyprids attaching in a month decreased to a minimum in 1945 and then rose once more. This fluctuation is more clearly indicated in Table II, in which the number of monthly cyprid totals greater than 1000 are compared with the number of monthly totals less than 1000 for six-month periods. The number of monthly cyprid totals of less than 1000 reached a maximum in the period from March 15, 1945 to September 1, 1945. The concurrence of a high proportion of monthly cyprid totals less than 1000 with a period of maximum barnacle collections suggests that conditions for survival and maturity of the cyprid were optimum when the daily rate of attachment totaled 400 to 1000 per month (Fig. 4, upper half).

TABLE II

*Number of monthly cyprid totals greater and less than 1000 in six-month periods*

| Period | >1000 | <1000 |
|---|---|---|
| March 15, 1943–September 1, 1943 | 9 | 3 |
| September 15, 1943–March 1, 1944 | 9 | 3 |
| March 15, 1944–September 1, 1944 | 6 | 6 |
| September 15, 1944–March 1, 1945 | 4 | 8 |
| March 15, 1945–September 1, 1945 | 2 | 10 |
| September 15, 1945–March 1, 1946 | 9 | 3 |

However, there were five occasions when the monthly cyprid totals in this range were not paralleled by monthly barnacle collections indicative of good survival. Since three of these collections were in the summer and two in the winter, it was not possible to identify these anomalies with a particular temperature condition or any other specific phenomenon. Cyprid totals of 2000 or more, which were found in 25 out of 74 monthly summations, invariably resulted in high mortality and poor survival. These data have been graphically presented by Deevey (1947) in a recent review of natural populations of animals. The phenomenon of attachment of enormous numbers of cyprids and high mortality was attributed by Moore (1935) to depletion of the stock of food in the water by the extra-large population. It should be pointed out that there were several occasions, particularly in 1945, when monthly cyprid totals slightly greater than 1000 were paralleled by large barnacle collections.

The ratio of the monthly totals of barnacles to cyprids is presented in the lower half of Figure 4. As shown, a ratio of 1 indicates survival of all attaching cyprids, and values less than 1 indicate the degree of survival (and mortality) of the cyprid population. In addition, there is the anomalous condition of ratios greater than 1, which indicates more adult barnacles at the end of a month than the total of settling cyprids in the same period.

The survival ratio was greater than 1 in October 1943, May and December 1944, and on nine of the monthly collections in 1945. A satisfactory explanation of this unusual condition may be found in the thesis of Phelps (1942) and of Scheer (1945) that the bacterial slime film, which forms on surfaces submerged in the sea, provides an environmental condition more suitable for attachment and growth than a clear bare surface. The daily collector, wiped clean every morning, may not have been as attractive a surface for attachment as the collector continuously immersed, which permitted a slime film to form within a few days after immersion.

During the period of observation, a distinct tendency toward greater survival of the cyprids was observed, reaching a peak in 1945 and then decreasing sharply. Although occasional rises of the barnacle-cyprid ratio were indicative of good survival in 1943 and 1944, the year 1945 was by far the best survival year.

Another factor limiting the survival of barnacles was the area available for growth on the collecting surface. The maximum adult population, one month old, was obtained during February 1945 when 1615 barnacles filled the collector. On many other occasions, particularly during the summer months when the growth rate was highest (Weiss, 1948), considerably fewer barnacles, for example 525 in June 1945, filled the same collecting area. Thus the area available for barnacle attachment was limited by the rate of growth of the barnacles initially attached.

## SUMMARY

1. The daily barnacle cyprid attachment in Biscayne Bay, Florida, and concomitant water temperature and salinity measurements were recorded continuously during a period of 38 months.

2. No relationship between the frequent peaks in cyprid settling and changes in temperature and salinity was established.

3. Seasonal periods of heavy cyprid attachment were observed to occur within a temperature range from 18° C. to 27° C.

4. The survival of cyprids increased gradually from 1943 to 1945 and then decreased sharply, as shown by a comparison of monthly barnacle collections with monthly totals of daily cyprid attachments.

5. Seasonal variation in growth rates was observed to be an important factor in limiting the numbers of barnacles attached per unit area.

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# A STUDY OF THE REPRODUCTIVE ORGANS OF THE COMMON MARINE SHRIMP, PENAEUS SETIFERUS (LINNAEUS)

JOSEPH E. KING[1]

## INTRODUCTION

In connection with the investigations of the common commercial shrimp, *Penaeus setiferus* (Linnaeus), carried on by the U. S. Fish and Wildlife Service, studies have been made of the structure of the reproductive organs and accessory sex characters and the general nature of the reproductive process.

This report gives information on the anatomy and histology of the male and female sex organs, the maturation of the germ cells, and certain observations on the phenomena of impregnation and ovulation.

This study was undertaken to determine if there are structural characters within the gonads which might serve as an index to age and longevity and furnish information bearing on the frequency of spawning of an individual within a single breeding season. Such data are needed for estimating mortality rates and for an understanding of the reproductive potential of the organism.

## MATERIALS AND METHODS

The observations which follow are based upon the macroscopical and microscopical examination of the gonads and accessory organs of several hundred shrimp taken at irregular intervals over a period of several years. Because of various circumstances, it was necessary to obtain much of the material from the commercial catch. These shrimp had been iced on the fishing grounds immediately upon capture and after 8 to 10 hours were still in a satisfactory state of preservation for general histological study. Much of this material, of course, was not suitable for detailed cytological work. When opportunity permitted trips were made to the fishing grounds and fresh material obtained.

At the beginning of the study the entire gonads of several specimens of each sex were serially sectioned and examined to determine if the various parts of the organ were homogeneous in structure and stage of development of the germ cells. As in general they were found to be so, samples, thereafter, were taken of only a portion of the gonads. In the female, this was usually the abdominal extension of the ovary lying in segments XVII and XVIII. In the male two or three lateral lobes of the testis were preserved from each specimen, together with one entire vas deferens and terminal ampoule.

Two generally different microscopical techniques were employed: in the first, tissues were prepared for general histological study; in the second, ovarian material was treated so as to preserve its true chemical nature for cytological study. In the first method, the tissues were fixed in either Bouin's or Kahle's fluid, imbedded in paraffin and stained with Delafield's Hematoxylin (Harris modification) and Eosin (1 per cent in dioxane) or Mallory's triple connective tissue stain. In this method dioxane was used in the imbedding and staining operations.

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In the second method, the tissues were fixed in 20 per cent formalin, sectioned with a freezing microtome and stained with one of the following fat-specific stains: Sudan IV, Calco Oil Red, Calco Oil Blue or Oil Yellow OB. Sections obtained by this method were also treated with ninhydrin, a protein-specific microchemical reagent.

The sexes of *P. setiferus* are easily distinguishable. In the male the endopodites of the first pair of pleopods are modified to form a copulatory organ, the *petasma* (Plate C, *2* and 3). The corresponding abdominal appendages of the female do not show this modification but are similar to the other pairs of pleopods. Additional but less obvious differences between the sexes include the locations of the openings of the genital ducts, the possession by the female of a ventral thoracic structure, the *thelycum* (Plate A, 3), the slight modification of the second pair of pleopods in the male (Plate C, 4), and significant differences in total length attained. As an example of the latter, total length measurements made on April 15, 1942, of a random sample of 200 adult shrimp of the offshore commercial catch gave an arithmetic mean of 165 mm. for 110 males and 171 mm. for 90 females. The males ranged in length from 147 to 178 mm. and the females from 151 to 188 mm., with the corresponding modes at 165 mm. and 176 mm.

In the Penaeid shrimps, as contrasted with the Pandalidae (Berkeley, 1929), no cases of sex reversal have been noted. The spawned eggs are released free in the water and are not carried on the pleopods of the female as in most other Decapods.

## The Female Reproductive System

### *Gross Anatomy*

The female reproductive system (Plate B, 2 and Plate A, 2) consists of paired ovaries and oviducts and a single thelycum. The ovaries are partly fused, bilaterally symetrical bodies extending in the mature animal for almost its entire length, actually from the cardiac region of the stomach to the telson. In the cephalothoracic region each organ bears a slender anterior lobe and in most cases seven finger-like lateral projections. A pair of lobes, one from each ovary, extend the length of the abdomen.

The anterior lobes lie closely applied to the esophagus and cardiac region of the stomach. The lateral lobes are located dorsally to the large mass of hepatopancreas and ventrally to the pericardial chamber. The heart rides like a saddle over this portion of the gonad. The abdominal extensions lie dorso-lateral to the intestine and ventro-lateral to the dorsal abdominal artery.

Fortunately for the observer in the field, maturation is accompanied by distinctive color changes in the ovary as well as changes in its size, which make it possible for one to identify the stage of development with fair accuracy without microscopic examination. The ovaries of the young shrimp (Plate D, 5) are so small in size and transparent in nature that their dissection in the fresh specimen is particularly difficult. Such a shrimp is recorded as *U* for undeveloped.[2] As the shrimp grows,

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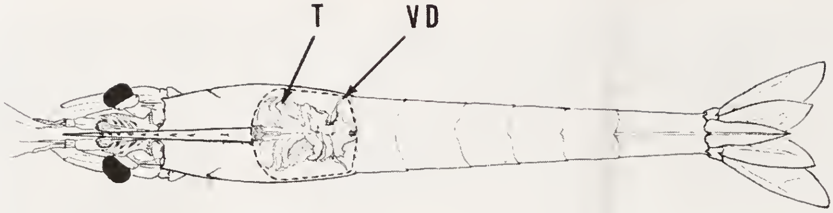
[2] Early in the history of the Investigations it was decided that the symbols *U*, *D*, *Y*, *R* and *Sp* were to be used by field workers in designating the obvious stages of ovarian development.

PLATE A



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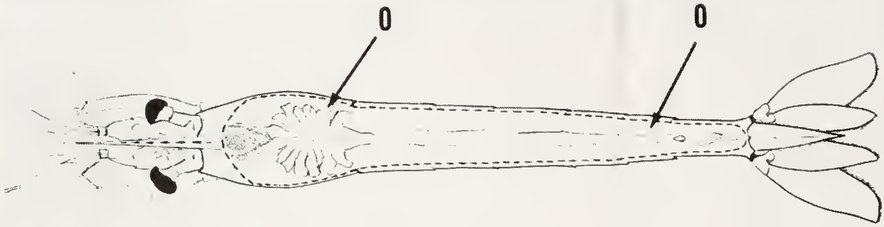

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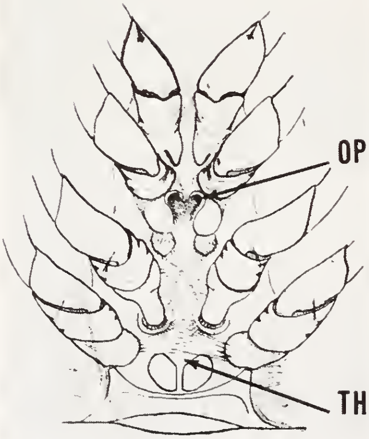

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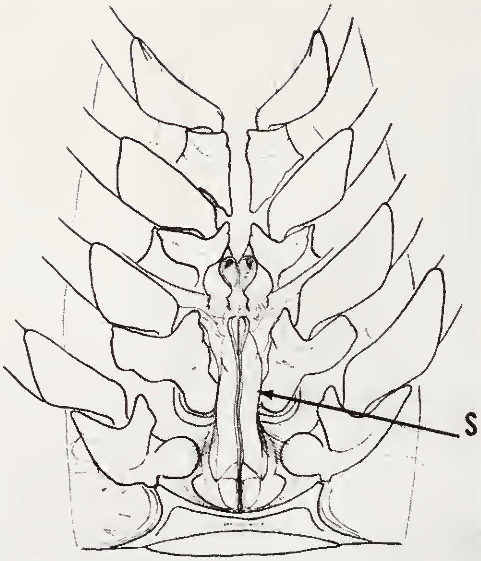

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the glands increase in size, scattered melanophores appear over the surface, and in general appearance, the ovaries become somewhat opaque. This stage is classed as *D,* or developing (Plate D, 3 and 4). As maturation continues, the ovary takes on a yellowish cast which deepens to a yellowish-orange, and is designated as the *Y*, or yellow stage (Plate D, 2). This is followed by the drab olive-brown color of the ripe, or *R* ovary (Plate D, 1). The ovaries, at this point, are so distended that they now fill all available space and appear to crowd considerably the other organs of the body cavity. During the yellow and olive-brown color phases, the ovary can be seen quite clearly through the abdominal tergites and membraneous connectives between the first abdominal somite and the carapace. The recently spawned ovary, designated as spent (*Sp.*), has collapsed from its distended condition and is not as deeply colored as in the ripe phase. As regression continues, the green color disappears but the structure remains opaque. A shrimp in this stage is difficult to distinguish from the large shrimp of the O-year class with developing ovaries. Microscopical examination, however, reveals the difference in most cases.

The oviducts (Plate B, 2) are short, narrow tubes leaving the ovaries at the tips of the 6th pair of lateral lobes and opening through the genital pores at the bases (coxae) of the 3d pair of pereiopods (Plate A, 3). Each opening is concealed in a bristle-filled, ear-shaped protuberance.

From a survey of the literature (Andrews, 1911; Burkenroad, 1934; Heldt, 1938; Filbo, 1943) it appears there are several ideas as to exactly what constitutes the thelycum (Plate A, 3). In *P. setiferus* it might be described as modifications of the sternal surfaces of somites XII, XIII, and XIV, which provide for the attachment of the spermatophore received from the male during copulation. In this species there are principally three things responsible for the retention of the spermatophore:

1. When discharged by the male, the spermatophore is accompanied by a mass of glutinous material (Plate B, 4 and 5) which is an important factor in its adherance to the female.
2. On the coxae of pereiopods four and five of the female there are protuberances bearing clumps of stiff bristles (Plate A, 3 and 4) which are directed medially and overlap flanges of the spermatophore, thus helping to hold it in place.
3. The "wings" of the spermatophore are securely anchored in a groove in the sternal surface between the 3d and 4th pereiopods (Plate A, 4). The horn-like structure on the posterior margin of each wing appears to be inserted between the bristle-bearing protuberance of the coxae of the 4th pereiopod and a raised shelf-like portion of sternite XIII.

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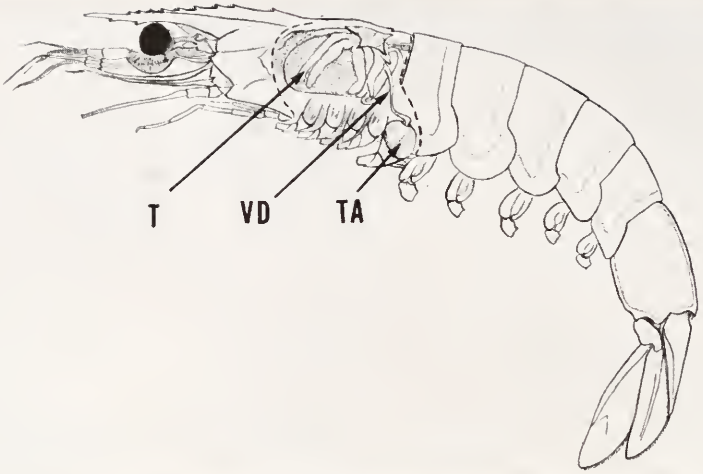
PLATE A

1. Diagram of male, dorsal view, dissected to show testes and portions of the vasa deferentia. T—testis; VD—vas deferens. × 0.5.
2. Diagram of female, dorsal view, dissected to show ovaries. O—ovary. × 0.5.
3. Diagram of ventral surface of cephalotharax of female. OP—opening of oviduct; TH—thelycum. × 2.5.
4. Diagram of ventral surface of cephalotharax of female with spermatophore attached. S—spermatophore. × 2.5.

PLATE B



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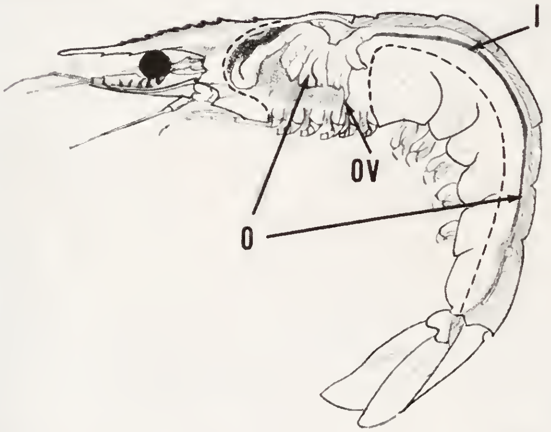

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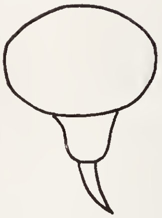

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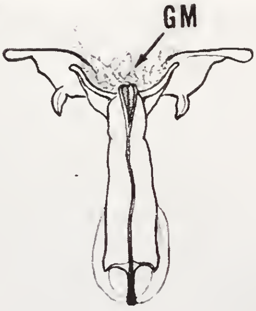

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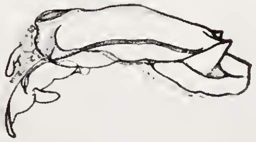

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On somite XIV are a pair of light-colored, pad-like structures which in *P. setiferus* play no active role in the impregnation process. In most other Decapods, however, these structures are open at their anterior margins, thus forming pockets that function as seminal receptacles.

Despite these holdfast devices, the spermatophore is rather easily dislodged, which may, to a certain extent, explain why spermatophore-bearing females are not commonly taken (Weymouth, Lindner and Anderson, 1933).

## *Histology and Development*

### *The ovary*

The wall of the ovary is composed of three layers: a thin outer layer of pavement epithelium, a relatively thick layer of connective tissue, and an inner layer of germinal epithelium. No layers of muscle fibers could be detected with the Mallory's triple staining technique. Extending longitudinally throughout the ovary are septa, also of connective tissue. Small blood sinuses can be seen in most cross-sections.

Germinal epithelium, functioning as such and giving rise to oogonia, is not distributed uniformly over the inner wall of the ovary but is confined to a certain well-defined area which has been termed the "zone of proliferation" (Gutsell, 1936). In the abdominal extension of the ovary there is a streak of such germinal epithelium along the medial-ventral wall of each lobe (Plate D, 1, 2, and 3; Plate E, 2, 3, and 4). In the lateral lobes this tissue occurs in a more strictly ventral position.

As the oogonia pass through the developmental stages of primary oocyte and secondary oocyte, they move in a column from the "zone of proliferation" toward the center of the ovarian lobe. Because of the rapid multiplication of cells, from this point the oocytes are forced to the peripheral regions of the lobe. As development proceeds, the peripheral and more mature oocytes are surrounded with "nurse" or follicle cells (Plate E, 3) which appear to arise from the germinal epithelium. Follicle formation continues until, in the ripe ovary, all the mature ova are enclosed by these nutritive cells.

The cytoplasm of the young ova, of *U* and *D* ovaries, appears finely granular with the staining methods used. As the egg matures these fine particles become enlarged and globule-like. In the *R* ovum we find very prominent rod-like bodies (Plate D, 6) imbedded in the cytoplasm in the peripheral regions of the cell and arranged radially about the nucleus.

With the Hematoxylin-Eosin staining technique, the cytoplasm of the *U* and *D* ova takes on a blue color indicating a basophilic reaction. As the egg matures into

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PLATE B

1. Diagram of male, lateral view, dissected to show reproductive organs. T—testis; VD—vas deferens; TA—terminal ampoule. × 0.5.

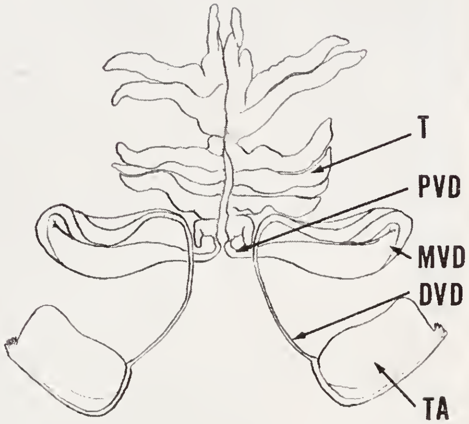
2. Diagram of female, lateral view, dissected to show relationship of ovary and oviduct. O—ovary; OV—oviduct; I—intestine. × 0.5.

3. Diagram of shrimp spermatozoan. × 9000 (approx.).

4. Diagram of ventral view of spermatophore (as in attached position). GM—gelatinous material. × 2.75.

5. Diagram of lateral view of spermatophore. × 2.75.

## Plate C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PVD
MVD
DVD
TA

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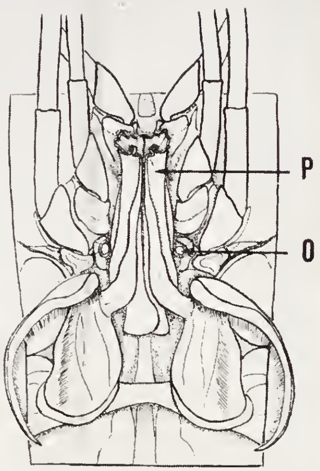

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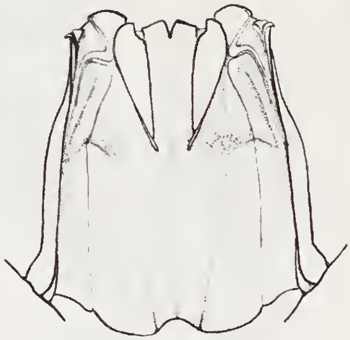

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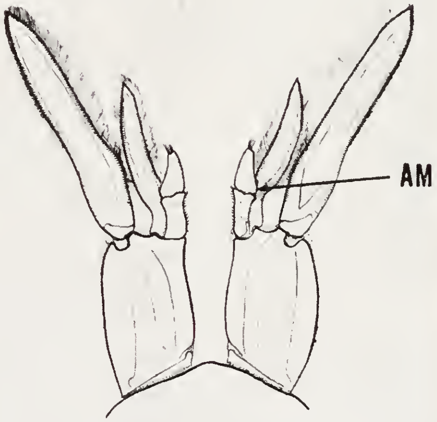

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the *Y* and *R* stages, the cytoplasm becomes acidophilic, staining red with Eosin. The rodlike peripheral bodies just described also stain red with Eosin.

Through the use of fat-specific dyes, such as Sudan IV, Calco Oil Red, Calco Oil Blue and Oil Yellow OB, it was found that no fat was present in the *U* ovaries, a very little in the more advanced *D* ovaries, and a great amount in the *Y* and *R* stages. The presence of fatty yolk in significant quantities coincides, therefore, with the appearance of the yellow color in the ovary.

In the ripe ovum the yolk globules reacted strongly with all of the fat soluble dyes used. The peripheral bodies, however, did not take on the dye, but appeared as distinct clear areas in the cytoplasm. It was concluded, therefore, that they are not of fatty nature.

Sections of ripe ova were treated also with ninhydrin, a protein-specific reagent, to determine if the rod-like bodies might be albuminous yolk. No reaction was obtained. Bhatia and Nath (1931) have observed very similar cell characters in the ova of *Palaemon lamarrei*, another species of shrimp. They believed the bodies to be albuminous yolk derived from mitochondria.

The recently spawned ovary is easily distinguishable from the ripe stage. It is flaccid rather than turgid, of a muddy green color, and microscopical examination reveals numerous ripe eggs undergoing resorption. As the degenerative process continues the gland becomes greatly reduced in size and assumes an opaque milky color. At this time it is very difficult to distinguish from an ovary in a late *D* stage. By taking into consideration such factors as total length, weight, and time of year, it is usually possible to segregate these groups in the field. When examined microscopically it is found that the two ovaries have a distinct differentiating character. The spent ovary is fairly swarming with left-over follicle cells (Plate D, 8). In the late *D* stage (Plate E, 3) follicle formation is just beginning and these cells are present only in small quantities.

A careful study of the spent ovary has been made to determine if it might be used as an index to the shrimp's age and number of times spawned. Although space in the ripe ovary is almost entirely given over to the crop of mature eggs, the "zone of proliferation" is still evident and a few young oocytes are continually being formed. Spent ovaries, three to four months after spawning, have undergone considerable regrowth. Even as early as March spent ovaries have been examined which showed evidence of regrowth. On this basis it appears quite possible for a shrimp spawning in March to spawn again later in the season. In view of the long spawning season, which in Louisiana offshore waters is March through September (Anderson, Lindner and King, 1948a), sufficient time may remain for the second crop of eggs to mature. We have been unsuccessful, however, in finding

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PLATE C

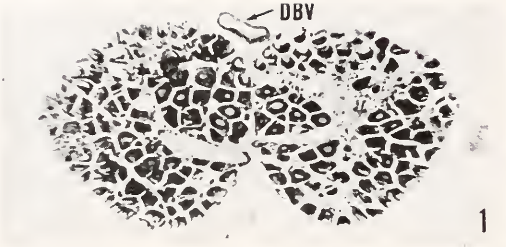
1. Diagram of male reproductive system. T—testis; PVD—proximal vas deferens; MVD—medial vas deferens; DVD—distal vas deferens; TA—terminal ampoule. × 1.75.

2. Diagram of ventral surface of mature male. P—petasma; O—opening of vas deferens. × 1.75.

3. Diagram of petasma of mature male spread open to show interior arrangement of folds. × 2.9.

4. Diagram of second pair of pleopods of male. AM—appendix musculina. × 1.75.

PLATE D



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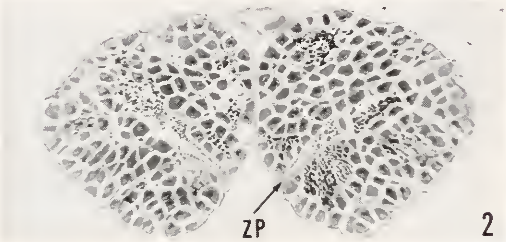

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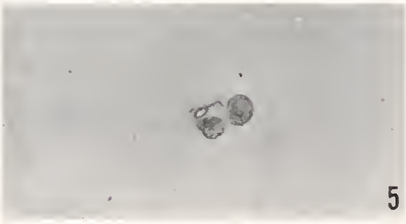

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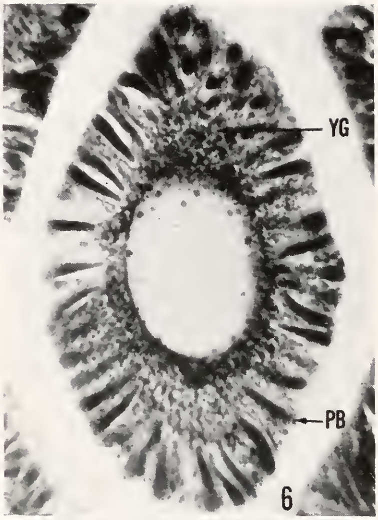

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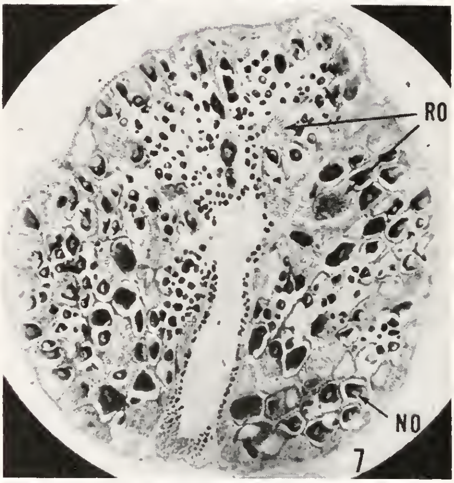

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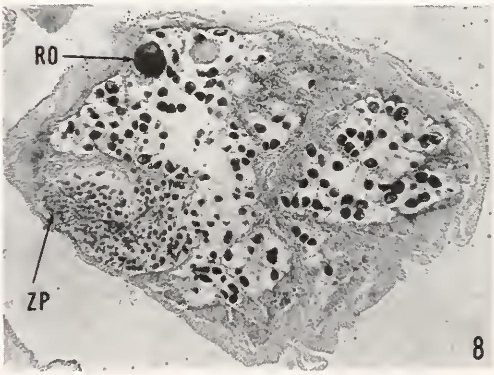

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any ovarian characters such as are present in the higher animals which would enable us to distinguish between the once-spawned and the twice-spawned ovary.

It was hoped that the structure of the ovary would supply clues as to the approximate age and length-of-life of the shrimp. This possibility was removed by the likelihood of multiple spawning and the absence of any permanent "scars" or walled-off spent areas within the ovary resulting from each spawning. For our knowledge of the longevity of the shrimp, therefore, we have had to rely upon information obtained through length frequency studies (Anderson, Lindner and King, 1948b, 1948c). These data have shown that young-of-the-year shrimp are dominant in the commercial catch after August of each year. By October and November, old of the previous year are very scarce and form a small percentage of the commercial catch. It is possible to follow through a trace of this old group until about the middle of January, after which they either die out or become merged with the following year class so that, with our present information, they cannot be distinguished (Lindner, Anderson and King, 1948). By the middle of January these old shrimp are approximately 1½ years of age. If shrimp live beyond this point, they are at least not found in the commercial catch much beyond December and not at all beyond the middle of January.

### *The oviduct*

The wall of the oviduct (Plate E, 6) appears to be made up of three layers. Bordering on the lumen is a somewhat folded columnar epithelium which, it is assumed, secretes a lubricating fluid to facilitate the passage of eggs down the tube. The main supporting structure of the duct is a thick layer of connective tissue. Although not apparent in cross-section, the outer layer would consist of a thin epithelial membrane. No muscle tissue was shown to be present by the Mallory's triple staining technique.

## *Ovulation*

It does not appear that nature has made the proper provision for the egress of the half million or so eggs which the average female will produce. As mentioned

### PLATE D

1. Ripe (R) ovary from 195 mm. shrimp taken March 7, 1944, cross-section of abdominal lobes. DBV—dorsal blood vessel. × 14.

2. Yellow (Y) ovary from 200 mm. shrimp taken March 7, 1944, cross-section of abdominal lobes. ZP—zone of proliferation. × 14.

3. Developing (D) ovary from 191 mm. shrimp taken December 15, 1944, cross-section of abdominal lobes. × 14.

4. Early developing (D) ovary from 180 mm. shrimp taken December 15, 1944, cross-section of abdominal lobes. × 14.

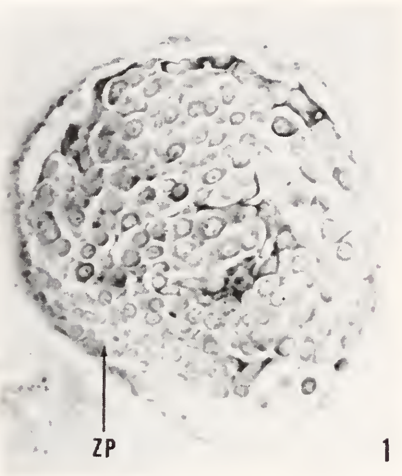
5. Undeveloped (U) ovary from 112 mm. shrimp taken November 15, 1944, cross-section of abdominal lobes. × 14.

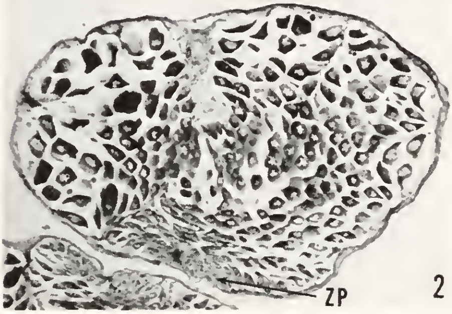
6. Ripe ovum, showing nucleus, yolk globules and rod-shaped peripheral bodies. YG—yolk globule; PB—peripheral body. × 279.

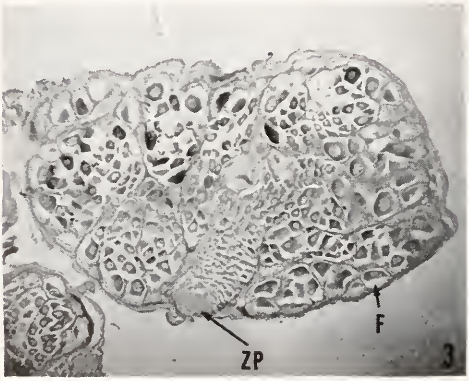
7. Spent (Sp.) ovary, cross-section of abdominal lobe of recently spawned shrimp. New crop of ova being produced. NO—new ovum; RO—resorptive ovum. × 35.

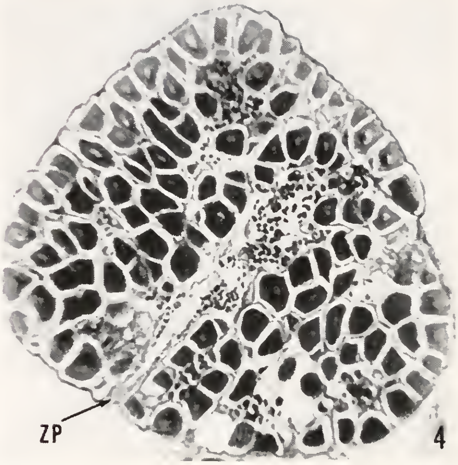
8. Spent (Sp.) ovary, cross-section of abdominal lobe showing almost complete recovery. Ovary is largely filled with dense masses of follicle cells and new oocytes. RO—resorptive ovum; ZP—zone of proliferation. × 52.

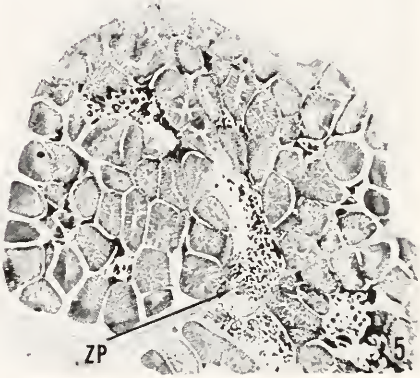
# PLATE E

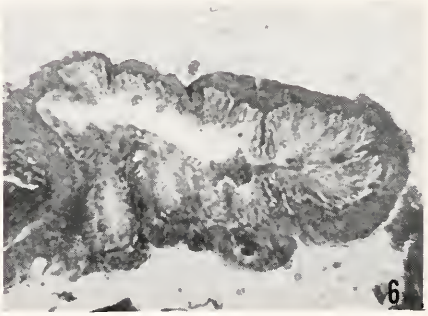












above, the oviduct is a slender though doubtless distensible tube connected with the ovary at the tip of one of the lateral lobes. No evidence was found of any branches of the oviduct extending beyond its junction with the ovary. In the ripe stage the ovary is very compactly filled with apparently no passageway through which the eggs might be directed to the mouth of the oviduct.

As the ovarian wall itself contains no muscle tissue, spawning is most likely brought about through the coordinated contracture of the cephalothoracic and abdominal muscles surrounding the ovary. It is probable that pressure is applied in such a way as to cause the eggs to move generally in the direction of the opening of the oviduct. Heldt (1938) observed the spawning of *P. trisulcatus* in an aquarium. She states that the female while resting on the bottom of the container emitted the eggs in great abundance. The process was completed in a few minutes.

As previously mentioned, a study of the "spent" ovary of *P. setiferus* reveals a large number of unspawned eggs in various stages of resorption (Plate D, 7 and 8), thus indicating that the ovary is not emptied in the spawning process and that a certain percentage of eggs never reach the exterior to be fertilized. Contrary to this, Heldt (1931) reports that in the spawning of *Penaeus caramote* Risso the ovary empties completely in one or two minutes.

## THE MALE REPRODUCTIVE SYSTEM

### *Gross Anatomy*

The male reproductive system (Plate A, 1 and Plate B, 1) includes paired testes, paired vasa deferentia and a petasma. The testes are unpigmented, translucent organs occupying a position in the body cavity very similar to that of the ovaries in the female, with the exception, however, that the testes lack the greatly extended abdominal lobes possessed by the ovaries. Each testis has projecting from its main axis an anterior lobe, apparently six lateral lobes, and a single short posterior lobe. The main trunks are more or less united for their entire lengths. In the young shrimp the testes are extremely delicate, transparent structures which can be located and removed only with great difficulty. As the animal matures, the testes change little in appearance, except to grow in size and become somewhat opaque.

The vasa deferentia arise from the posterior margins of the main axes of the testes and open to the exterior through genital pores located medially on the coxae

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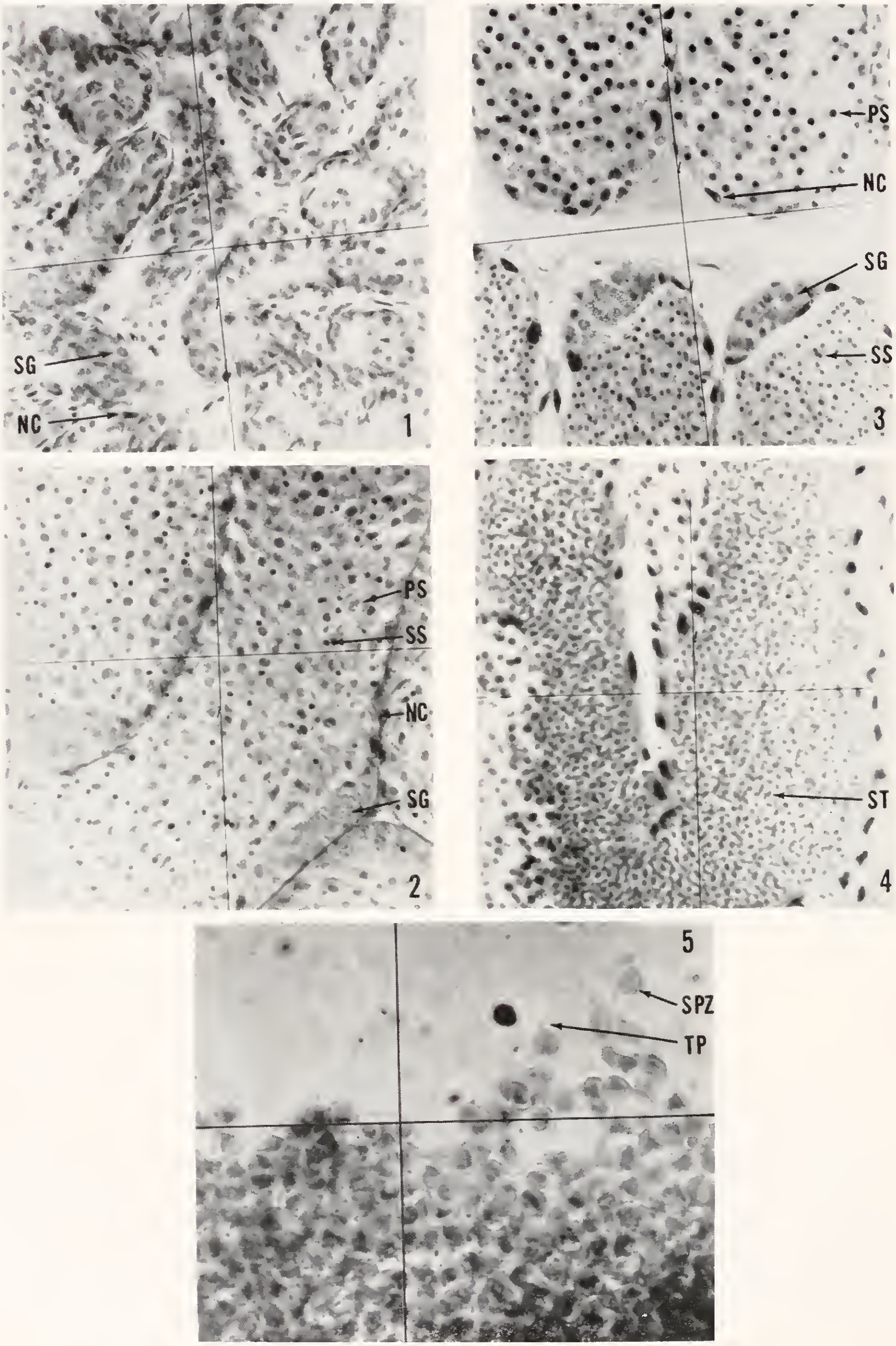
PLATE E

1. Undeveloped (U) ovary, cross-section of abdominal lobe. ZP—zone of proliferation. × 170.
2. Early developing (D) ovary, cross-section of abdominal lobe. ZP—zone of proliferation. × 82.
3. Advanced developing (D) ovary, cross-section of abdominal lobe. ZP—zone of proliferation; F—follicle cell. × 46.
4. Yellow (Y) ovary, cross-section of abdominal lobe; cytoplasm of ova is filled with yolk globules which take on a deep eosin stain. ZP—zone of proliferation. × 24.
5. Ripe (R) ovary, cross-section of abdominal lobe; peripheral bodies present in ova; young oocytes still being produced. ZP—zone of proliferation. × 27.
6. Oviduct, cross-section, showing thick outer wall and inner lining of tall columnar epithelium. × 102.

PLATE F


SG
NC
1
PS
NC
SG
SS
3
PS
SS
NC
SG
2
ST
4
5
SPZ
TP

of the 5th pereiopods (Plate B, 1 and Plate C, 2). Each vas deferens consists of four distinct regions (Plate C, 1): (1) a short, narrow, proximal section passing abruptly into (2) a thickened medial portion having a double flexure which tapers to form (3) a relatively long narrow tube terminating in (4) a greatly dilated muscular region, the ductus ejaculatorius, or terminal ampoule. Here in this distal region is formed the spermatophore, the structure in which the sperm cells are conveyed from male to female.

The petasma (Plate C, 2 and 3) is a complicated membraneous structure of folds and troughs resulting from great modifications of the endopodites of the first pair of pleopods. The two symmetrical components are not fused but are held together in the mid-line by numerous, interlocking and very minute hooks, resembling a zipper in appearance and effect. The petasma supposedly functions in the transfer and attachment of the spermatophore to the ventral surface of the female.

In *P. setiferus* the endopodites of the second pair of pleopods are slightly modified to form the "appendix masculina," which may also assist to a small extent in the handling of the spermatophore.

The spermatophore is roughly pod-like in design (Plate B, 4 and 5), each terminal ampoule contributing a part, the halves being assembled outside the body immediately following their expulsion. The anterior end (as when in position on the female) of the spermatophore bears a pair of "wings" which are important in anchoring the structure in place. The posterior-dorsal region is extended to form a flange or shelf which also functions in attachment. It has been determined by dissection and by forcibly expelling the halves of the spermatophore with thumb and forefinger that each half is discharged from the terminal ampoule with the winged end foremost. And also that the right and left halves of the spermatophore, as in position on the female, are produced by the corresponding right and left terminal ampoules of the male, i.e., the right half originates in the right ampoule.

Never having observed the union of the sexes of the Penaeidae and being unable to find a description of the process in available literature, we can only attempt to imagine how this transfer must take place. It is assumed that the halves of the spermatophore are discharged almost simultaneously and with the aid of the walking legs are fitted together and placed in the clasping-like trough on the ventral surface of the petasma. In view of the origin and final orientation of the spermatophore, it appears necessary that the male and female should assume a head-to-tail position with their ventral surfaces in close proximity, in order to effect the transfer of the spermatophore. It is possible, however, that the petasma does not play as im-

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## PLATE F

1. Section of immature testis containing spermatogonia and follicle cells. SG—spermatogonium; NC—nutritive cell. × 289.

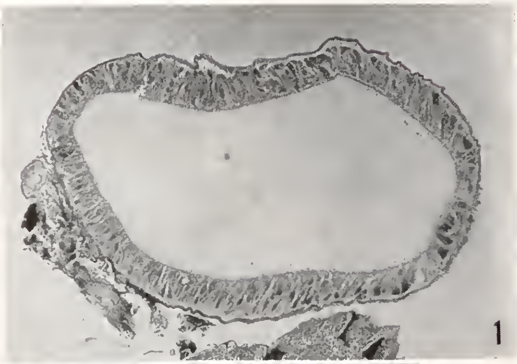
2. Section of mature testis, each tubule containing spermatogonia, primary spermatocytes and secondary spermatocytes. SG—spermatogonium; PS—primary spermatocyte; SS—secondary spermatocyte; NC—nutritive cell. × 289.

3. Section of mature testis, primary spermatocytes and secondary spermatocytes in separate but adjoining tubules. SG—spermatogonium; PS—primary spermatocyte; SS—secondary spermatocyte; F—follicle cell. × 289.

4. Section of mature testis, showing spermatids. ST—spermatid. × 289.

5. Spermatozoa from medial region of vas deferens. SPZ—spermatozoan; TP—tail piece. × 850.

## Plate G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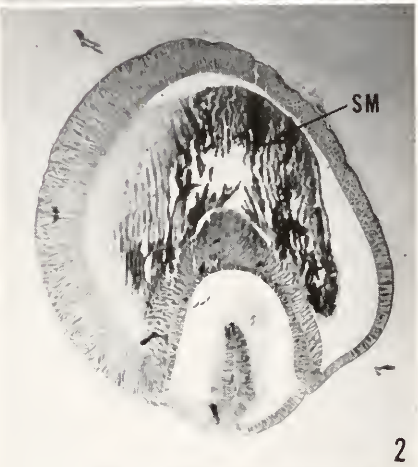

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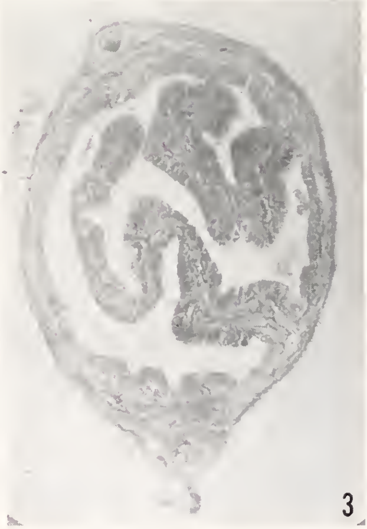
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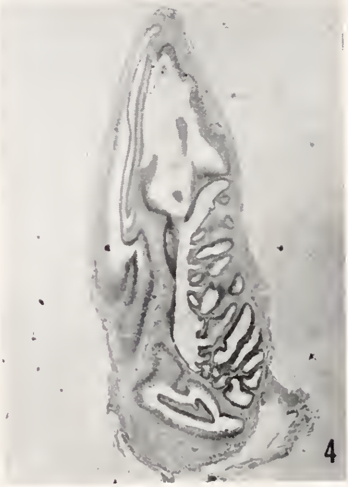
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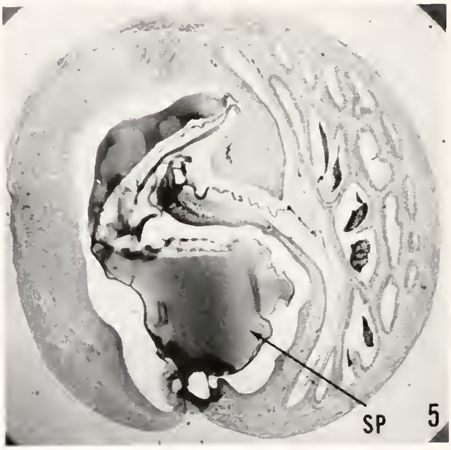

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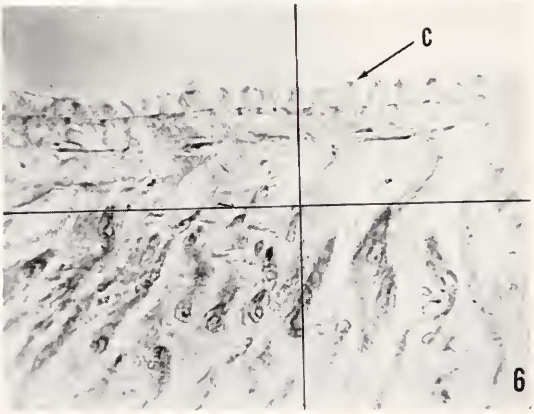

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portant a role as is generally assumed and that the walking legs may possess sufficient dexterity to manipulate the spermatophore so as to effect its attachment with the male and female in a head-to-head position.

When fixed in position on the female (Plate A, 4), the anterior end of the spermatophore is in close proximity to the openings of the genital pores. The factors coordinating the dehiscence of the spermatophore with the expulsion of the eggs are as yet unknown; however, it may be that the fluid accompanying the eggs has a chemical or physical effect causing the spermatophore to open and release the sperm at the appropriate moment.

## *Histology and Development*

### *The testis*

The thin wall or cortex surrounding the testis consists of two layers: an outer epithelium, and an inner layer of connective tissue. No muscle tissue is present. The body of the testis is composed of a mass of very minute, convoluted, seminiferous tubules in which the male reproductive cells are produced. It appears that the membrane-like wall of each tubule is also of two layers: the outer tunica or membrana propia, and the inner germinal epithelium.

The content of the tubules varies with the size of the shrimp and also with the season. For example, the testes of a young 130 mm. shrimp in February are much farther advanced than the testes of a 130 mm. shrimp in November. The tubules of small shrimp were found to contain spermatogonia and scattered nutritive or nurse cells (Plate F, 1). The spermatogonia have fairly definite cell walls and large round nuclei surrounded by a distinct mass of protoplasm. The nurse cells have irregularly-shaped nuclei imbedded in a syncitial mass of protoplasm with no visible cell boundaries.

As the season advances and the shrimp grows, the testes and the individual tubules increase greatly in size. Each spermatogonium passes through a period of quick growth to become a primary spermatocyte. A reduction division results in two secondary spermatocytes. These divide again to produce four spermatids which develop without further divisions into spermatozoa. The primary spermatocytes are larger than the secondary spermatocytes (Plate F, 2 and 3), which are in turn larger than the spermatids (Plate F, 4). In tubules containing these later stages, the nurse cells appear to be confined to the peripheral region of the tubule.

Once a shrimp reaches sexual maturity, the testes produce a continuous crop of sperm cells. At almost any time of the year an examination of the mature testis will reveal germ cells in all stages of development. Most of the tubules in cross-

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PLATE G

1. Vas deferens, cross-section, in proximal region. × 49.
2. Vas deferens, cross-section, in medial region, showing refractory sperm mass and extremely tall columnar epithelium lining cavities. SM—sperm mass. × 22.
3. Vas deferens, cross-section, in distal region. × 88.
4. Terminal ampoule, longitudinal-section, of immature shrimp. × 17.
5. Terminal ampoule, cross-section, of mature shrimp; spermatophore in process of formation. SP—spermatophore. × 14.
6. Cilia-bearing epithelium from terminal ampoule. C—cilia. × 367.

section show two typical stages but adjoining tubules may contain cells in different stages of development. The mature tubule (Plate F, 2) may normally contain along one margin a layer, several cells in thickness, of spermatogonia and primary spermatocytes. The main bulk of the tubule will be filled with secondary spermatocytes or spermatids or both.

### *The spermatozoa*

Ripe spermatozoa were observed only in the vas deferens. The spermatozoan (Plate B, 3 and Plate F, 5) is composed of three typical parts: head, middle piece, and tail. The head is large and almost circular in outline, the middle piece is short and considerably more slender, while the tail is relatively thick and short. From its structure it is a logical assumption that the spermatozoan is capable of movement.

### *The vas deferens*

The vas deferens leaves the testis as a simple tube (Plate G, 1) lined with cilia-bearing columnar epithelium and with some muscle and connective tissue in its outer wall. As the diameter of the tube increases, a longitudinal septum appears in its interior separating the tube into two channels of unequal diameter (Plate G, 2). The sperm cells move down the larger channel, their flow doubtlessly being facilitated by the beating of cilia and by secretions of the glandular epithelium. Within the smaller channel there develops from the margin a longitudinal fold which may be traced distally to the terminal ampoule. The epithelium lining the smaller channel produces a fluid which is probably involved in the formation of the spermatophore. This secretion and also the sperm mass become very hard and brittle when passed through the usual fixing and imbedding procedures, thereby making the sectioning process difficult.

After the second bend the vas deferens is reduced in diameter to form a narrow tube (Plate G, 3) which joins with the terminal ampoule. At the beginning of the narrow portion, the longitudinal septum becomes detached from the wall at one of its margins, forming a shelf or fold which continues to the terminal ampoule and parallels the other fold previously described. With the severing of this partition, the sperm-bearing fluid and the secretion of the other channel may come in partial contact for the first time. The arrangement of the folds, however, would tend to prevent any extensive intermingling of the two substances.

The greatly dilated distal region, or terminal ampoule (Plate G, 4 and 5), possesses a thick muscular wall lined with extremely tall columnar epithelial cells. From our observations the detailed composition of the wall is as follows: first a thin outer squamous epithelium underlain with connective tissue, several bands of circular muscles, then a wide zone which appears to be composed of interspersed longitudinal and circular muscle fibers, another layer of connective tissue, and finally the thick glandular epithelium. The latter contains enormous nuclei and bears cilia in certain areas. The numerous folds and partitions are formed by extensions of columnar epithelium supported by connective tissue.

In cross- or longitudinal sections the terminal ampoule of the mature shrimp presents a very complex picture. Examination of these organs from a number of individuals showed, however, that the general arrangement was quite uniform for all. The most easily identifiable structures of the developing spermatophore are the sperm cells enclosed in a sheath and surrounded by a mass of chitinous material. In some sections the wings of the spermatophore may be seen in process of formation. Because of the refractory nature of the contents of the ampoule following fixation and imbedding, it is extremely difficult to obtain good sections for study.

Fasten (1917) has called attention to the fact that cilia have been considered universally absent from the Arthropoda. He found them, however, to occur in the vasa deferentia of Anomura and Brachyura. As reported here, striking examples of these processes are found also in the male tract of the Peneidea.

## SUMMARY

The general anatomy and histology of the reproductive organs of the shrimp, *Penaeus setiferus* (Linnaeus), have been studied and described.

Although the cytological phenomena of oogēnesis and spermatogenesis have not been completely worked out, gross developmental changes in size and coloration of the organs have been related to histological and cytological changes so that the field worker, without access to a microscopy laboratory, may more accurately determine and record the stage of development.

Observations have been included on the processes of impregnation and ovulation.

Unsuccessful efforts were made to find clues which would serve as an index to the age of a shrimp. Evidence was found, however, indicating that a female may spawn more than once in a single breeding season.

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# ROTATORIA FROM PENIKESE ISLAND, MASSACHUSETTS, WITH A DESCRIPTION OF PTYGURA AGASSIZI N. SP.

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This paper is based on material collected from the three main ponds on Penikese Island, Massachusetts on August 3 and 17, 1947. The collections were made in connection with the celebration of the 75th anniversary of the founding by Louis Agassiz of the Anderson School of Natural History. A large group of biologists visited the island for the purpose of making a commemorative biological survey. I am indebted to my wife who did much of the collecting and sorting of material.

Penikese Island is near the end of the chain of Elizabeth Islands leading southwest from Woods Hole, at latitude 41° 27′ N. and longitude 71° 51′ W. All three ponds were small and shallow, but Typha Pond was nearly dry. This pond was, however, nearly fresh, while the other two contained considerable concentrations of salt. While this paper is limited to aquatic species of rotifers, those living in terrestrial moss were collected by Mr. Andre Burger of Neuchâtel, Switzerland, and will be reported by him (1948).

All the species listed are widely distributed and have been reported from salt water before, with the exception of the two species of *Ptygura*.

## LIST OF SPECIES

Tub Pond, August 3, 1947. Salinity 34 ‰
*Brachionus plicatilis* Müller

Typha Pond, August 3, 1947. Salinity 5 ‰
*Brachionus calyciflorus* Pallas
*Cephalodella catellina* (Müller)
*Colurella obtusa* (Gosse)
*Lecane bulla* (Gosse)

South Pond, August 3 and 17, 1947. Salinity 15 ‰
*Collotheca ornata* (Ehrenberg)
*Colurella colurus* (Ehrenberg)
*Lecane closterocerca* (Schmarda)
*Lecane grandis* (Murray)
*Ptygura agassizi* n. sp.
*Ptygura crystallina* (Ehrenberg)

## NOTES ON SPECIES

### *Cephalodella catellina* (Müller)

There has long been some uncertainty about the proper terminology of the *catellina* group of species. Since Müller's original figure cannot certainly be associated with any one of the species, it is necessary to take Ehrenberg's description as the first adequate definition, and the basic issue then becomes the identity of the form called *catellina* by Harring and Myers with Ehrenberg's species. The specimens found in Typha Pond agree well with those described as *catellina* by Edmondson and Hutchinson (1934) or *myersi* in Wiszniewski's careful study (1936).

### *Colurella colurus* (Ehrenberg) (Fig. 11)

The few specimens collected agree fairly well with Lucks' description of *compressa* (1912), synonymized with *colurus* by Harring (1913). However, they were considerably more elongate than any *colurus* yet described. Since the genus is in need of a complete revision, no discussion is attempted here.

### *Lecane grandis* (Murray)

The specimens in general agree well with Murray's description (1913). There seems to be some variation in the degree to which the coxal plates project beyond the ventral plate of the lorica. The lateral spines were absent from the toes of one specimen, but this character is known to be variable in other species of the genus.

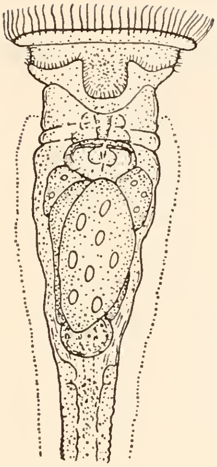
### *Ptygura crystallina* (Ehrenberg) (Fig. 10)

One specimen was found which appeared to be a rather young *P. crystallina* (Fig. 10). The dorsal antenna was considerably more conspicuous than usual, and it is not entirely certain that this is indeed *crystallina*. The tube was fully formed and, in addition, there was a thin gelatinous layer closely adhering to the cuticle of the body. Such a covering has not been observed on specimens in fresh water.

### *Ptygura agassizi* n. sp. (Figs. 1–9)

The corona is broadly elliptical with a very narrow dorsal gap and no ventral notch (Fig. 3). Its plane is at a fairly large angle to the axis of the body, in some positions looks almost perpendicular (Fig. 8). The buccal area is large relative to the corona, about half as long as the vertical axis. The cuticular areas lateral to the buccal area are wide and flattened, giving a characteristic appearance to the head in oblique views. The neck is about as long as deep. The body is about four-fifths as wide as the corona, fusiform, widest near the anterior end and tapering gradually to the foot. The dorsum has five or more prominent wrinkles, the most anterior of which encircles the body and separates it from the neck. The foot is heavily wrinkled and is usually about one and a half or two times as long as the rest of the body, but is very extensible and in active specimens may be three times as long. The peduncle is a short cylinder considerably narrower than the foot.

The lateral antennae are minute setate cylinders near the ventral side of the neck. On the dorsal side of the neck is a small sharply pointed spine. No setae were observed on it and it is not now possible to decide whether the spine represents a rudimentary dorsal antenna, or whether that organ is absent altogether, and this spine is comparable to that of *Ptygura melicerta* var. *mucicola*. A number of mature adults retained the eyes near the dorsal side of the neck (Fig. 2), but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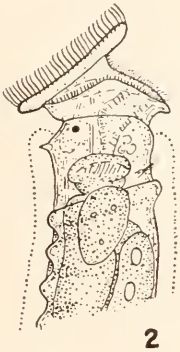
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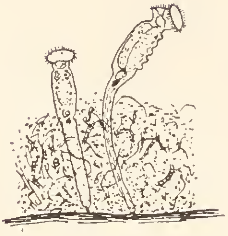
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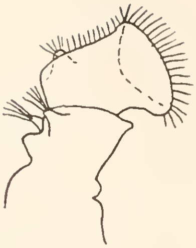
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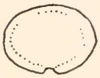
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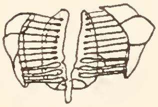
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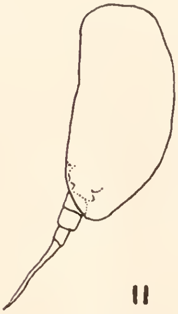
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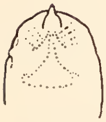
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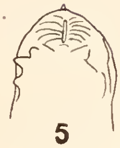
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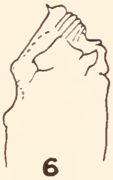
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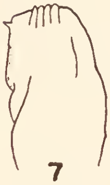
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Explanation of Figures

Figure 1. *Ptygura agassizi,* ventral view.
Figure 2. *Ptygura agassizi,* lateral view.
Figure 3. *Ptygura agassizi,* corona.
Figure 4. *Ptygura agassizi,* anterior end, fully contracted, dorsal view.
Figure 5. *Ptygura agassizi,* same specimen, ventral view.
Figure 6. *Ptygura agassizi,* same specimen, lateral view.
Figure 7. *Ptygura agassizi,* anterior end, partly contracted, ventral view.
Figure 8. *Ptygura agassizi,* two specimens showing characteristic attitude.
Figure 9. *Ptygura agassizi,* trophi, right manubrium partly folded down.
Figure 10. *Ptygura crystallina,* lateral view.
Figure 11. *Colurella colurus,* lateral view.

this was not true of all. The trophi are of the usual type. The first three teeth of the unci are definitely larger than the others.

The tube was difficult to observe because the Ruppia to which the animals were attached was covered with a layer of flocculent material containing bacteria and many capsulated algae. Apparently the tubes of the rotifers were short, irregularly shaped and made of rather loose gelatinous material. In addition to the usual tube, the animals were covered by a thin gelatinous layer adhering closely to the cuticle, shown by dotted lines in Figures 1 and 2. This cannot be taken as a specific character, for *P. crystallina* also had such a sheath; possibly this is an effect of salinity.

Dimensions of a typical specimen: total length, 480 $\mu$; foot length, 285 $\mu$; corona width, 90 $\mu$.

*Ptygura agassizi* resembles in a general way *P. melicerta,* particularly variety *mucicola.* It differs in the shape of the dorsal spine and lack of any other structure corresponding to the dorsal antenna. The details of the shape of the corona and buccal area are different, particularly in the angle of the plane of the corona to the axis of the body and in the flattened areas at the sides of the buccal area. Moreover, *P. agassizi* does not have the rigorous substrate limitations of *P. melicerta* (Edmondson, 1944). One of the specimens corresponded especially closely, except for dorsal spines, with Hudson and Gosse's description of *Oecistes serpentinus,* generally regarded as a synonym of *P. melicerta* (Harring, 1913); it was particularly active in contracting foot and corona, had a poorly shaped, small tube, and contained oil globules in the posterior part of the body as did a number of the other specimens. The external morphological characters agreed with those given above. The only other members of the genus which seem to have much resemblance to this are *P. tihanyensis* and *P. stephanion,* both of which differ from *agassizi* in the corona, and lack of a dorsal spine.

This species occurred in some abundance in South Pond, and is named for the zoologist whose memory was being honored by the biological survey of the island. The type has been deposited in the American Museum of Natural History, and a paratype in the Yale Peabody Museum.

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# THE EFFECT OF NITROGEN MUSTARDS ON THE RESPIRATION AND FERTILIZATION OF SEA URCHIN SPERM AND EGGS[1]

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The halogenated alkyl amines, the so-called nitrogen mustards, are extremely interesting compounds to the biologist. When dissolved in water they give quaternary nitrogen transformation products which in small amounts inhibit mitosis (Friedenwald and Schultz, 1943; Bodenstein, 1947) and produce mutations (Auerbach *et al.,* 1943; Miller and McElroy, 1948). These transformation products act as structural inhibitors for those enzymes where choline is the substrate in the enzyme-substrate complex. Furthermore, nitrogen mustards act as inhibitors of other enzymes by virtue of the great reactivity of their ethylene immonium derivatives and of their halogen groups. All these enzyme inhibitions produce inhibition of tissue respiration and of synthesis reactions (Barron, Bartlett and Miller, In press; Barron, Bartlett, Miller, Meyer and Seegmiller, In press). The effect of nitrogen mustards on cell division of fertilized sea urchin eggs was studied by Cannan and Levy (1944) and Vislocki *et al.* (1945), who found that they produced a delay in the rate of cellular division. As continuation of the work of this laboratory on the mechanism of action of nitrogen mustards, the effect of these compounds on the respiration and fertilization of sea urchin eggs and sperm was studied. Nitrogen mustards were found to produce in sea urchin sperm a striking stimulation of respiration accompanied by an inhibition on fertilization of eggs of the normal sequences of cell division and development.

## EXPERIMENTAL

Male and female species of *Arbacia punctulata* were allowed to shed their sperm and eggs. The eggs were washed in a large amount of filtered sea water and the excess water was withdrawn previous to the experiments. The sperm were suspended in twelve volumes of filtered sea water, shaken, centrifuged for five minutes, and resuspended in the desired amount of sea water. The nitrogen mustards (their HCl salts) were dissolved in sea water just before use and were added to the sperm suspension 15 to 30 minutes previous to the measurement of respiration. The manometric experiments were performed at 25° C.; the fertilization experiments, at the room temperature of the laboratory, 23°–25° C.

*Effect of nitrogen mustards on the respiration of sperm and of eggs of sea urchin.* Freshly dissolved methyl bis ($\beta$-chloroethyl) amine HCl (MBA) at a concentration of 0.001 *M* added to sperm suspensions from 15 to 30 minutes be-

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TABLE I

*Effect of methyl bis (β-chloroethyl) amine HCl (MBA) on the respiration of sea urchin sperm*

The cells were suspended in sea water. Temperature 25° C.

| Experiment No. | $O_2$ Uptake per Hour | | |
|---|---|---|---|
| | Control | MBA | Increase |
| | c. mm. | c. mm. | Per cent |
| I | 46.6 | 128.8 | 176 |
| II | 56.5 | 139 | 146 |
| III | 80 | 115 | 44 |
| IV | 41 | 84 | 105 |

fore measurement of the $O_2$ uptake increased it from 44 to 176 per cent (Table I). This variation in the increase of respiration is probably due to the different periods of times which elapsed from addition of MBA to the measurement of the $O_2$ uptake. In fact, when MBA was added while respiration was being measured, there was an induction period of about 30 minutes when the $O_2$ uptake continued at the same rate as before addition of MBA, the increase starting afterwards (Fig. 1). This induction period must be due to the slow formation in sea water of the active ethylene immonium transformation product (transformation of nitrogen mustards into the ethylene immonium derivative is greatly retarded in the presence of KCl and NaCl).

Increase in respiration was also shown on addition of other halogenated alkyl amines. In experiments performed under similar conditions, MBA increased the respiration of sea urchin sperm by 196 per cent; isopropyl bis (β-chloroethyl) amine HCl, 115 per cent; ethyl bis (β-chloroethyl) amine HCl, 155 per cent; and tris (β-chloroethyl) amine HCl, 79 per cent (Table II).

The increase in respiration was obtained with concentrations varying between 0.001 *M* and 0.0001 *M*. When the concentration of MBA was diminished to 0.00001 *M* there was no effect at all with sperm suspensions of 1:20. With concentrations of MBA of 0.0001 *M*, the increase in respiration, which did not start until one hour and a half after addition, remained constant for the duration of the experiment, eight hours. However, when the concentration was increased ten

TABLE II

*The effect of nitrogen mustards on the respiration of sea urchin sperm*

The nitrogen mustards (0.0001 *M*) were dissolved in sea water and added 20 minutes before measurement of the $O_2$ uptake.

| Nitrogen mustard | $O_2$ uptake | |
|---|---|---|
| | Control | N Mustard |
| | c. mm. | c. mm. |
| Methyl bis (β-chloroethyl) amine HCl | 42 | 124 |
| Isopropyl bis (β-chloroethyl) amine HCl | 42 | 90 |
| Ethyl bis (β-chloroethyl) amine HCl | 42 | 107 |
| Tris (β-chloroethyl) amine HCl | 42 | 75 |

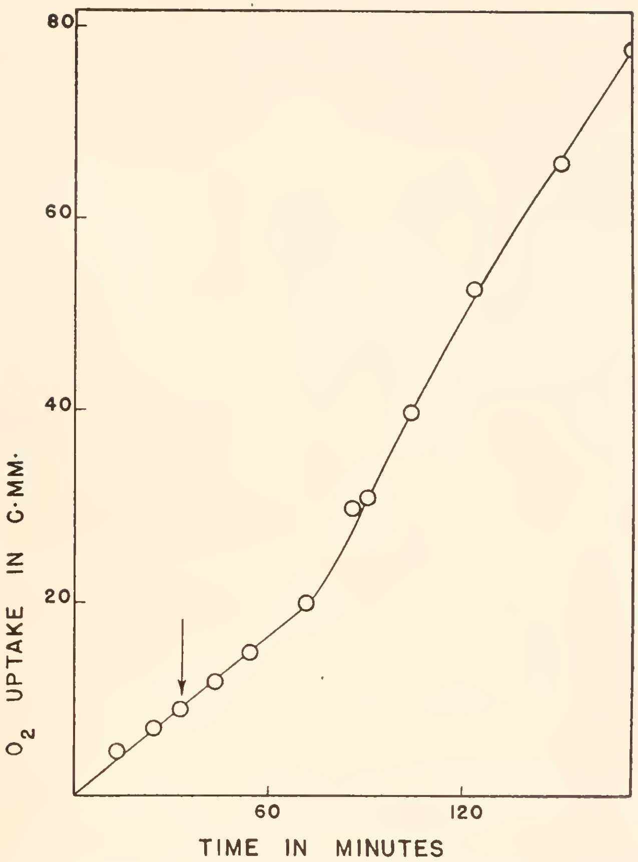

FIGURE 1. Effect of methyl bis (β-chloroethyl) amine HCl (0.001 *M*) on the respiration of sea urchin sperm. MBA was added at time marked by arrow. Temperature 25° C.

times (0.001 *M*) the increase in respiration diminished after three hours (Fig. 2). This increase of respiration followed by a diminution seemed to indicate that increase and inhibition could be obtained by either increasing the concentration of nitrogen mustard or by decreasing the concentration of sperm cells. The latter method was chosen to test this possibility. Sperm suspensions were diluted with sea water as follows: 1 of sperm and 10 of sea water; 1:50; 1:100 and 1:200. After addition of 0.001 *M* MBA, the $O_2$ uptake of the sperm suspensions was measured for nine hours. The most striking results were obtained on sperm suspensions diluted 1:200. In the first hour, MBA increased the respiration by 120 per cent; in the second hour this increase dropped to 54 per cent; and in the third hour it disappeared completely. In the fourth hour the inhibition started (27 per cent), to become complete in the sixth hour (Fig. 3). Sperm suspensions at this dilution had a remarkably high respiration, for the $Q_{O_2}$ of the control samples was 26.0; furthermore, the $O_2$ uptake remained at steady values throughout the duration of the experiments, nine hours.

TABLE III

*Effect of MBA (0.001 M) on the respiratory quotient of sea urchin sperm*

| Experiment No. | $O_2$ uptake | | $CO_2$ production | |
|---|---|---|---|---|
| | Control | MBA | Control | MBA |
| I | 54.5 | 171 | 50 | 147.5 |
| II | 62.0 | 164 | 70 | 140 |
| III | 76 | 137 | 75 | 128 |
| IV | 49 | 147 | 50 | 144 |

This increase seems to be confined to oxidative processes because nitrogen mustards had no effect on the anaerobic glycolysis of sea urchin sperm.

The increase in respiration seems to take place on the whole respiratory process as shown by measurements of the respiratory quotient (R.Q.). The R.Q. of sea urchin sperm is close to 1 as found by Barron and Goldinger (1941). The average R.Q. value of four experiments (triplicate determinations for each experiment) was 1.01 for the control samples. The R.Q. of the nitrogen mustard containing samples was 0.93 (Table III).

The respiration of sea urchin eggs whether fertilized or unfertilized was not affected by addition of MBA or isopropyl bis ($\beta$-chloroethyl) amine HCl (Table IV). This lack of effect cannot be due to lack of penetration for nitrogen mustards added to sea urchin eggs produce a delay in the cleavage of eggs when they are fertilized with normal sperm (Cannan and Levy, 1944; Vislocki *et al.*, 1945).

Inhibitions of enzymes and of tissue respiration produced by nitrogen mustards can be prevented on addition of thiosulfate or of choline (Barron, Bartlett and Miller, In press). Previous addition of these two substances to sperm at a concentration 50 times that of MBA had no effect at all on the increased respiration produced by the nitrogen mustard. The increase in respiration was not associated with any possible combination of seminal fluid with the nitrogen mustard (if we accept the postulated inhibition of respiration by seminal fluid), for stimulation of

TABLE IV

*Effect of MBA and isopropyl bis ($\beta$-chloroethyl) amine HCl on the respiration of sea urchin eggs*

| Nitrogen mustard (0.001 *M*) | $O_2$ uptake in 1 hour | | | |
|---|---|---|---|---|
| | Unfertilized eggs | | Fertilized eggs | |
| | Control | N. mustard | Control | N. mustard |
| MBA | 19.1 | 16.8 | 40 | 37 |
| MBA | 17.0 | 15.0 | 46 | 44 |
| Isopropyl bis ($\beta$-chloroethyl) amine HCl | 20 | 18 | 52 | 54 |
| Isopropyl bis ($\beta$-chloroethyl) amine HCl | 21 | 23 | 32.5 | 31.5 |

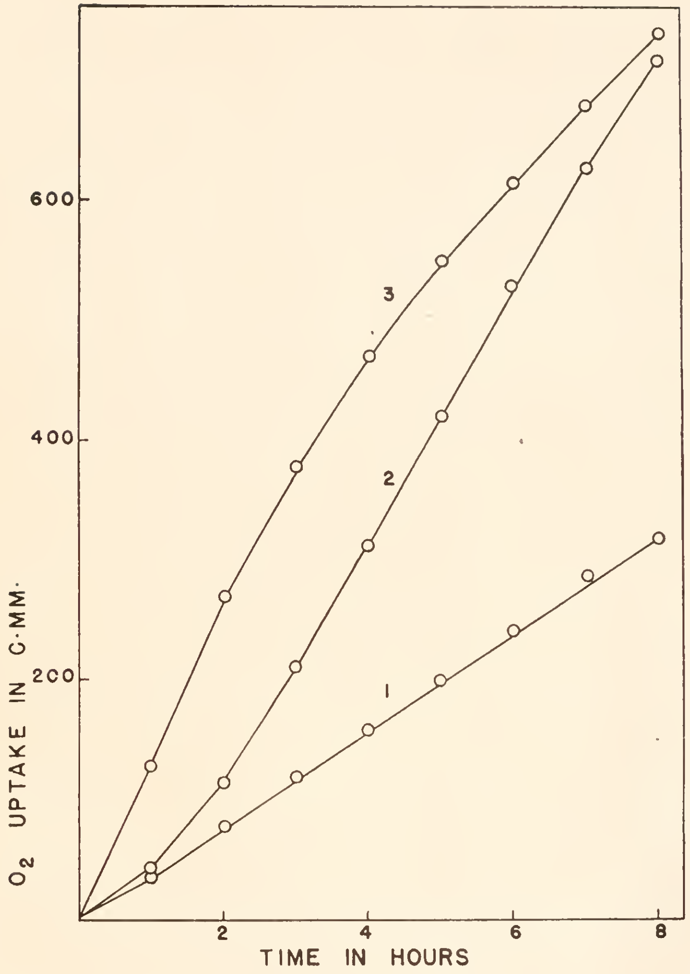

FIGURE 2. Increase in respiration produced by MBA on sea urchin sperm. Effect of concentration of MBA. 1. Control; 2. MBA, $1 \times 10^{-4}$ *M*; 3. MBA, $1 \times 10^{-3}$ *M*.

respiration was obtained on sperm suspensions washed repeatedly with sea water by successive suspensions and centrifugation.

*Effect of MBA on the fertilization of Arbacia eggs.* Cannan and Levy (1944) and Vislocki *et al.* (1945) found that nitrogen mustards in small concentrations produced a delay in the rate of cleavage of sea urchin eggs. In their experiments 0.0013 *M* MBA was added to a suspension of eggs, and 20 minutes later the eggs were washed and fertilized with non-treated sperm. Under those conditions, while first cleavage (50 per cent) took place in 56 minutes in the control eggs, it required 78 minutes in the MBA treated eggs. Treatment just before fertilization started retarded first division but not the second division. The inhibition of cell division was found more effective when the sperm was treated with nitrogen mustard, as can be seen in the two series of experiments.

*First series of experiments.* A suspension of sperm (1:50) was added to different concentrations of MBA to give the following concentrations: $1 \times 10^{-3}$ *M*; $1 \times 10^{-4}$ *M*; $1 \times 10^{-5}$ *M*; and $1 \times 10^{-6}$ *M*. Forty minutes later, 0.1 cc. of this sperm suspension was added to 20 cc. of sea water containing unfertilized eggs. During the first cleavage period the eggs receiving sperm suspensions with an initial MBA concentration of $1 \times 10^{-3}$ *M* and $1 \times 10^{-4}$ *M* (concentration in the egg suspension, $5 \times 10^{-5}$ and $5 \times 10^{-6}$) showed only 1 and 3 per cent of eggs in the two-cell stage. The eggs receiving sperm with an initial MBA concentration of $1 \times 10^{-5}$ *M* and $1 \times 10^{-6}$ *M* showed 75 per cent and 92 per cent cells in the two-cell stage. Thirty hours later, when the control cells were all motile, in the blastula and pluteus stages, the cells receiving sperm with MBA at the initial concentrations of $1 \times 10^{-3}$ *M* and $1 \times 10^{-4}$ *M* were all nonmotile; the cells receiving sperm with MBA concentration of $1 \times 10^{-5}$ *M* had 30 per cent of motile cells and those receiving sperm with $1 \times 10^{-6}$ *M* MBA had 50 per cent of motile cells. Furthermore a number of morphological alterations were observed (Table V).

TABLE V

*Effect of MBA added to sperm on the fertilization and development of sea urchin eggs*

The sperm was diluted 1:200.

| Time after fertilization | Control | Initial concentration of MBA, *M* | | | |
|---|---|---|---|---|---|
| | | $1 \times 10^{-3}$ | $1 \times 10^{-4}$ | $1 \times 10^{-5}$ | $1 \times 10^{-6}$ |
| Minutes | Per cent | Per cent | Per cent | Per cent | Per cent |
| 66—First cleavage<br>2 cell stage | 80 | 1 | 3 | 75 | 92 |
| 118—Second cleavage | | | | | |
| 2 cell stage | 18 | 65 | 85 | 20 | 18 |
| 4 cell stage | 75 | None | 3 | 74 | 82 |
| 180—2 cell stage | — | 4 | 2 | 2 | |
| 4 cell stage | 10 | 39 | 7 | 3 | 2 |
| Many cell stage | 90 | 37 | 87 | 95 | 98 |
| 25 hours—Blastula<br>Motile | 60 | 3 | 20 | 75 | 60 |
| 30½ hours—Motile<br>Blastula | All | None | None | 30 | 50 |

*Second series of experiments.* Suspensions of sperm were treated with $1 \times 10^{-3}$ *M* MBA and twenty minutes later 0.05 cc. of this suspension was added to a suspension of eggs in 5 cc. sea water. A suspension of unfertilized eggs was treated with an amount of MBA corresponding to that received by the eggs fertilized with MBA treated sperm; twenty minutes later they received normal sperm. At the end of 158 minutes, while the control and the treated eggs had 90 per cent of cells in the 4-cell stage, the eggs receiving treated sperm had only 39 per cent. Twenty-seven hours later the control and the MBA treated eggs had the same amount of

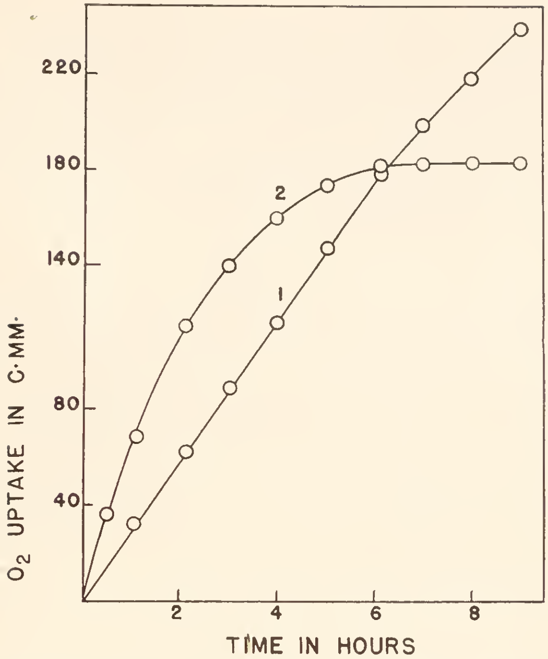

FIGURE 3. The effect of MBA (0.001 *M*) on the respiration of dilute sperm suspensions. Dilution, 1 : 200. $Q_{O_2} = 26.0$; 1. Control; 2. MBA.

actively moving pluteus (45 per cent) while the eggs receiving treated sperm had only 8 per cent of motile pluteus and had 16 per cent of cells morphologically abnormal.

Delay in the rate of cell cleavage, decrease in the number of cells reaching the pluteus stage, as well as abnormal cells, were found even when sperm and MBA were in contact for only two minutes before fertilization.

## DISCUSSION

The sperm of sea urchin is a striking exception in its response to the addition of nitrogen mustards. While other cells or tissues are either inhibited or not affected in their respiration, the sperm of sea urchin showed a marked increase. This increase, which comprises the whole respiration process there being no alteration in the respiratory quotient, must be due to the action of nitrogen mustards on the regulatory mechanisms of the cell, i.e., those mechanisms which control the equilibrium relationships between anabolic and catabolic processes. The increase in respiration seems to occur, however, only when small concentrations of nitrogen mustard penetrate into the cells, for when the cell concentration was decreased complete inhibition of respiration followed the initial stimulation. Undoubtedly, as more nitrogen mustard penetrated the cells a concentration was reached—enough to produce the known enzyme inhibitions found by Cori (1943), Dixon (1942) and Barron *et al.* (In press). The stimulation of respiration might be due to combination of the halogen groups of nitrogen mustard with soluble – SH groups in the sperm cell, groups which act as regulators of respiration. Nitrogen mustard produced also a profound impairment in the fertilization power of sperm. In fact, sperm treated with nitrogen mustard was more effective in inhibiting the rate of cell division of fertilized eggs. Furthermore, the eggs were stopped in their development so that none reached the pluteus stage. The mechanism of this inhibition is not yet known; it is undoubtedly related to the property of nitrogen mustards of inhibiting mitosis and of producing mutations.

## SUMMARY

The halogenated alkyl amines, methyl bis ($\beta$-chloroethyl) amine HCl, isopropyl bis ($\beta$-chloroethyl) amine HCl, ethyl bis ($\beta$-chloroethyl) amine HCl, and tris ($\beta$-chloroethyl) amine HCl, when added to sperm suspensions of *Arabacia punctulata* increased their respiration. This increase seems to affect the entire respiratory process as shown by the unaltered R.Q. value. Anaerobic glycolysis was not altered. With dilute sperm suspensions (1:200) the stimulation of respiration was followed by inhibition. Sperm so treated produced, on fertilization of eggs, an inhibition in the rate of cleavage, of pluteus formation, and motility of blastula, and produced in addition a number of malformations.

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# A STUDY OF THE CHROMOSOMES IN TWO SPECIES OF BATS (CHIROPTERA)[1]

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The Chiroptera is an order of mammals in which the chromosome survey has progressed less than in other orders and, despite their wide distribution all over the world, our cytological knowledge of them has remained incomplete. With reference to the evolution of mammals, the order Chiroptera occupies a very important position, since they are closely related to the Insectivora, which in turn are of interest in their relation to the Primates. In view of these facts a thorough exploration of the chromosomes of these mammals is highly desirable.

The literature pertaining to the chiropteran chromosomes is filled with discrepancies. Van der Stricht (1910), the first to concern himself with the chiropteran chromosomes, recorded the approximate number as 9–10 in the oocytes of *Vesperugo noctula*. Athias (1912) in an incomplete account of the chromosomes of *Rhinolophus hiposideros* and *Vesperugo serotinus* reported about 16 chromosomes as haploid in the oocytes of the former species and 15–24 in the latter. Jordan (1912) counted over 24 chromosomes in the diploid group of a male bat (the species name not given) and reported an XO chromosome mechanism. Working with a species of bat (the name unknown), Hance (1917) reported an approximate number of 40 for the male diploid complex. One is impressed from the above accounts that these earlier workers were handicapped by inadequate technique and any conclusive evidence cannot be expected from them. The only author who succeeded to some extent in surveying the morphology of chromosomes in this field is Painter (1925). He determined the diploid number of chromosomes in the embryonal cells of the horse bat, *Nyctinomus mexicanus,* to be 48, but presented no evidence relating to the maturation divisions.

The present paper deals with the chromosomes of the Okinawa fruit bat, *Pteropus dasymallus inopinatus* Kuroda, a member of the Megachiroptera, and those of the Japanese horse-shoe bat, *Rhinolophus ferrum-quinum nippon* Temminck, belonging to the Microchiroptera. In the former species, the material proved favorable for a thorough study of chromosomes throughout spermatogenesis, while in the latter form only the primary spermatocytes came under observation because of the unfavorable season for collecting the material. But in view of the unsatisfactory status of the chromosome survey in this order, the publication of even such a fragmentary account seems justified.

It is pleasant to express the author's sincere appreciation to Dr. Kan Oguma for valuable suggestions. Cordial thanks are also due to Professor Franz Schrader of Columbia University for his kind service in revising the manuscript and in its publi-

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cation. Gratitude is extended to Dr. Y. Yamashina and the late Dr. D. Nakamura through whose generosity the material for this study was made available. Financial aid from the 4th Special Committee for Researches of Genetics in the Nippon Gakuzyutsu-Shinkokwai, as well as from the Scientific Research Expenditure of the Department of Education is also acknowledged here.

### *The chromosomes of Pteropus dasymallus inopinatus Kuroda*

*Pteropus dasymallus inopinatus* Kuroda, commonly known as the Okinawa fruit bat, is a member of the Pteropodidae of the Megachiroptera. It is distributed throughout the Riu-Kiu Islands. Testicular material obtained from two specimens caught in different localities was employed for study. One of them came to hand through the generosity of Dr. Y. Yamashina and was killed in September 1940. This animal came from Okinawa and had been reared for a few years in the Yamashina Ornithological Institute, Tokyo. The other specimen was secured by the author at Nago, Okinawa in April 1941. The testes were removed in living condition and fixed in Flemming's solution with no trace of acetic acid. Subjected to the usual paraffin method, the sections were stained with Heidenhain's iron-haematoxylin and light-green. The observations were chiefly carried out on the former material, which was fixed at a more favorable season of the year.

Careful counting of several adequate metaphase figures of the spermatogonia gave 38 as the diploid number in every case. As shown in Figures 1 and 2, the chromosome complement of this form is remarkable not only for its comparatively low number, but also for the fact that the majority of the chromosomes are J- or V-shaped. Close examination reveals that, except for a pair of elongated rod-shaped elements together with a Y chromosome of minute size, all the other elements are provided with atelomitic fibre attachments. The latter are submedian or subterminal in location. The autosomes vary in size, ranging from enormous J- or V-shaped elements to small, slightly curved bodies. In the equatorial plate, the latter lie with the bend directed towards the centre of the plate and with their long axes stretching across the radius of the equatorial plate.

The two elongated chromosomes, in turn, are in striking contrast to the others due to their remarkable rod-shape. They form a homologous pair, identical in length, and seem to be of telomitic nature, since they have tapering ends at their inner extremities where the spindle fibres attach. They correspond in length to one arm of the largest V-shaped chromosome.

The even number of chromosomes herein established naturally suggests the occurrence of an XY-pair of sex chromosomes, as is the general rule in the mammals so far reported. On account of its size and shape, the Y chromosome is readily distinguishable from the others. The identification of the X element, however, presents some difficulty. It is probable that the X is represented by one of the atelomitic chromosomes. To judge from the morphology of the sex chromosome pair as disclosed in the first meiotic division, the X element seems to be represented by one of the submedian chromosomes, fairly large in size and having extremely dissimilar arms.

The chromosomes found in the primary spermatocyte metaphase are observable with extreme clearness due to the reduced number of elements. The chromosome count at this stage is therefore made with certainty and thus it gives final confirmation of the diploid number obtained in the spermatogonia. Every metaphase plate under observation shows consistently 19 chromosomes, having various shapes and sizes (Figs. 3 to 5). The chromosomes are all bivalent in structure. The haploid complex consists of 18 autosomal bivalents with ordinary structure and an XY complex of heteromorphic nature, composed of the large J-shaped X and the small Y.[2] Corresponding to the morphology of the spermatogonial chromosomes, the autosomal bivalents are variable in size and the majority of them appear as atelomitic in their fibre attachment. Three or four of these bivalents are relatively small in size, in striking contrast to the remaining ones. Among the larger bivalents the size differences are slight and they form a closely graded series.

The XY-bivalent is readily distinguishable from the autosomal bivalents because of its unusual asymmetrical configuration, and by its relatively peripheral position in the metaphase plate (XY in Figs. 3 to 5). It consists of the J-shaped X element of large size and the very minute Y element. The latter is attached to the extremity of the long arm of the X. At metaphase, the X is placed so that its longer arm with the Y at its extremity is vertical to the equatorial plate, while the shorter arm lies in most cases parallel to the equatorial plate. Sometimes the longer arm of the X acquires two constrictions due probably to the elongation, giving as a result a somewhat undulating appearance (Figs. 6 and 7). To judge from the structural configuration, the spindle fibres of the X attach to the point where the long and short arms join, while in the Y they apparently attach to its free end. The whole configuration displayed by the XY bivalent, as outlined in the above description, is clearly displayed in the profile view of the metaphase plate of the primary spermatocyte, as given in Figures 6 to 8.

At the commencement of anaphase of the first division, the separation of autosomes takes place synchronously and there is usually no element which lags or precedes the others (Fig. 8). During the division of the autosomal elements, the X also disjoins from the Y and they migrate to opposite poles. The anaphasic picture is clearly seen in Figure 9.

There are discernible two kinds of secondary spermatocytes, though each has an equal number of chromosomes, resulting from the reductional segregation of the X and Y in the first division. The one consists of 18 autosomal elements and the X chromosome (Fig. 10), and the other contains a corresponding set of autosomal elements and the minute Y (Fig. 11).

### *The chromosomes of Rhinolophus ferrum-equinum nippon Temminck*

The Japanese horse-shoe bat, *Rhinolophus ferrum-equinum nippon* Temminck, is a common species of the Rhinolophidae (the Microchiroptera) and known throughout Hokkaido, Hondo, Shikohu and Kyusyu. The testes of a male specimen which was obtained at Nagasaki in October 1937 were fixed with Flemming's solution without acetic acid by the late Dr. D. Nakamura. After imbedding in paraffin the material was sent to the author. Staining was done with Heidenhain's iron-haematoxylin and light-green.

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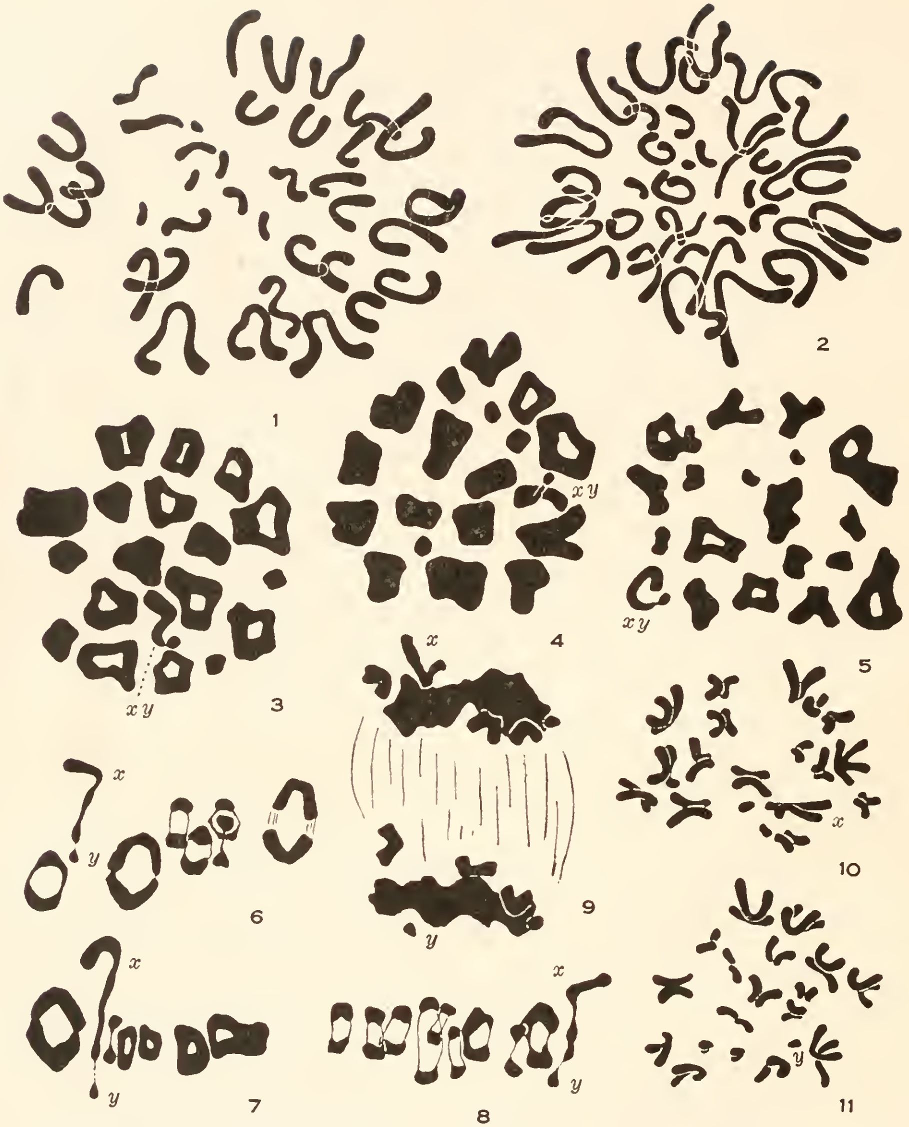
[2] It will be understood that since no female material was available, the specific identification of the X and Y chromosomes is tentative. However, in view of the general agreement in the configuration of the sex chromosomes of other mammals, there would appear to be small doubt about its correctness.

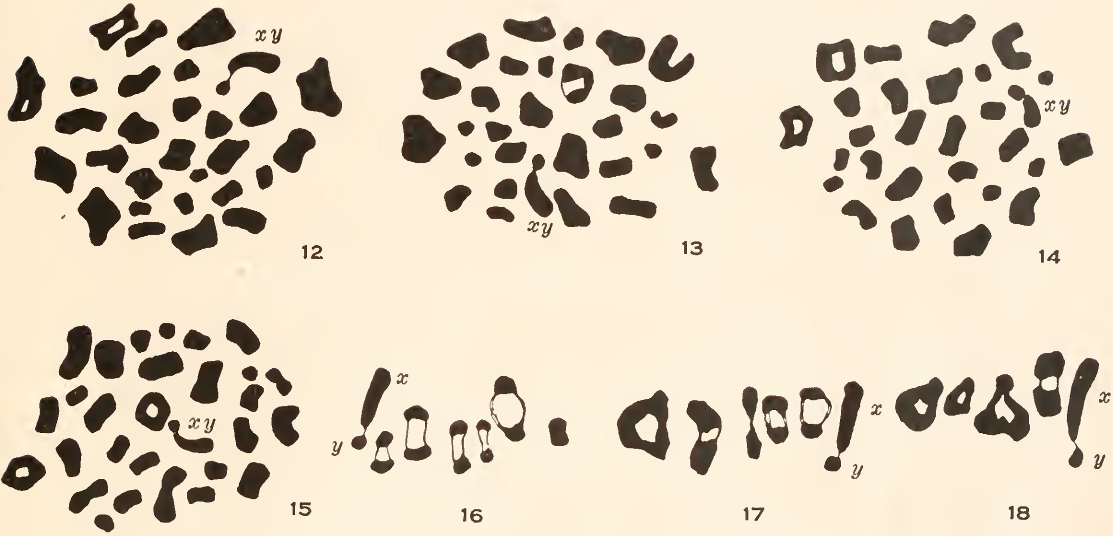


All drawings at a magnification of 3200 × (reduced from 6000 ×)

FIGURES 1–11. Chromosomes of *Pteropus dasymallus inopinatus.* 1–2, Spermatogonial metaphases, showing 38 chromosomes. 3–5, primary spermatocyte metaphases, showing 19 bivalents. 6–8, side views of the first division metaphases, showing the XY complex. 9, side view of the first division anaphase, showing the segregation of the X and Y elements. 10, secondary spermatocyte metaphase, showing 19 dyads. X-class. 11, the same, Y-class.

The material proved to be insufficient for a complete study of the spermatogenesis, because of the unfavorable season of collection; consequently only fragmentary notes concerning the primary spermatocyte chromosomes can be given here.

The haploid number of chromosomes was determined in the primary spermatocyte. The author was surprised at finding a higher number of chromosomes in this species than that encountered in the previous form. Counts in several adequate plates established that there are 29 distinct chromosomes having bivalent nature. As seen in Figures 12 to 15, the chromosomes vary considerably in their size and shape. The configuration of some of the larger bivalents is strongly suggestive of subterminal or submedian fibre attachments. To the author's view, a few of the medium sized ones seem also to be of atelomitic nature. Four or five bivalents are extremely small in size. Outstanding in the haploid complement is a heteromorphic bivalent which is composed of a long rod-shaped element and a minute granular one, connected end to end with each other. Taking all characteristic conditions into consideration, it is concluded that this particular chromosome configuration is the XY-bivalent, the larger component being the X and the smaller the Y (XY in Figs. 12 to 15). The XY-bivalent usually takes a peripheral position in the metaphase arrangement.



All drawings at a magnification of 3200 × (reduced from 6000 ×)

FIGURES 12–18. Chromosomes of *Rhinolophus ferrum-equinum nippon*. 12–15, primary spermatocyte metaphases, showing 29 bivalents. 16–18, side views of the first division metaphases, showing the XY complex.

It is in the side view of the metaphase spindle that the configuration and structure of the XY-bivalent are most clearly observed (Figs. 16 to 18). Morphologically considered, the X seems to be provided with a telomitic fibre attachment. The Y element is more or less spherical in outline and seems to be slightly smaller than the smallest autosome. The two chromosomes are connected to each other, end to end, by a fine fibre and are placed nearly perpendicularly to the equatorial plate in a linear configuration. The mode of conjugation as seen in the X and the Y is fairly comparable to that found in the mice as reported by the author (Makino, 1941).

On the basis of the above results, the following statement is possible: the diploid number of chromosomes in this species would appear to be 58, comprising both atelomitic and telomitic elements of varying size and shape. The sex chromosomes are of the usual XY type, the X having an elongated rod-shape and the Y a minute dot-like one.

## REMARKS

It seems to the author that among the earlier works on chiropteran chromosomes the only account sufficiently accurate to warrant a more detailed discussion is that of Painter (1925) on the house bat, *Nyctinomus mexicanus,* a species of the Molossidae. Painter (1925) made the chromosome count in the amnion of young embryos and established 48 chromosomes as the diploid number of this species. Of these, at least six pairs of chromosomes have a distinct V-shape (Painter, 1925, Plate I). In comparison with the present material, it is thus evident that the number of chromosomes of *Nyctinomus* is smaller than that of *Rhinolophus ferrum-equinum nippon,* in which 29 haploid chromosomes were clearly counted. However, no precise comparison is possible with the data at hand.

The present communication constitutes the first report on the chromosomes of one of the Macrochiroptera. As reported in the foregoing pages, the diploid number of chromosomes of the Okinawa fruit bat, *Pteropus dasymallus inopinatus,* is 38. Referring to the literature on mammalian chromosomes (see the list newly compiled by Makino, 1947), it is apparent that the chromosome number here established for this species is rather low in comparison with that of other eutherian mammals, so far as the recorded cases are concerned. Furthermore, specially remarkable is the fact that the karyotype of this form is probably archaic among the eutherian species so far studied, since, excepting one pair of long rod-shaped autosomes and the Y chromosome, the chromosomes are all provided with an atelomitic fibre attachment, the majority of them being V- or J-shaped and of large size.

No accurate and clear-cut evidence has been demonstrated for the sex chromosome mechanism of the Chiroptera in any of the previous investigations. Painter (1925) working with *Nyctinomus,* was unable to observe the sex chromosome of this species in the maturation division. He stated that the constancy of the chromosome number in different embryos together with the fact that in some embryos a pair of unequal sized elements is observed, would seem to indicate that we have in this form the XY type of sex chromosome. The evidence presented in the present study shows that the XY mechanism of sex determination occurs in both of the species under study. In both the X and the Y separate in the first division, and pass to opposite poles.

Painter (1925) counted the chromosome number in 10 species of eutherian mammals covering 7 different orders, and emphasized that 48 chromosomes occur in the most primitive eutherian order of Insectivora, and in such divergent orders as the Primates and Chiroptera. He deduced from this evidence that the chromosome number of 48 seems to be the typical and is probably the basal number for the mammals.

During recent years, the present author has engaged in a comparative study of mammalian chromosomes, endeavoring to make as broad a survey of Eutheria as possible. At present his investigations have extended to 42 species which represent 1 species of the Insectivora, 2 species of the Chiroptera, 22 species of the Rodentia, 4 species of the Carnivora, 8 species of the Artiodactyla, 2 species of the Perissodactyla, 1 species of the Cetacea and 2 species of the Primates. Some of the results have already been reported (Makino, 1942, 1943 a, b, c, 1944 a, b, c, 1946, 1947 a, b). The results of these studies demonstrate that there is a striking diversity in the number of chromosomes of the species studied, ranging from 30 in the Vole, *Microtus kikuchi* to 78 in the dog, *Canis familiaris,* and further that the chromosome number of 48 was proved to exist in only four out of 42 species studied—to wit—*Buballus buffelus* of the Artiodactyla, and *Apodemus agrarius, A. semotus* and *Lepus gichiganus ainu* of the Rodentia. Thus the newly obtained data clearly point to the fact that the occurrence of 48 chromosomes is relatively rare in mammals, and can no longer be regarded as the basal number. It is evident that our knowledge of the chromosomes of mammals is still too scanty to justify any generalization. To discover the basal number and to establish a phylogenetical relationship on the basis of cytology is a task which will require the accumulation of accurate data in an immense number of closely related forms, before one can attempt to formulate any rule of general application.

## SUMMARY

The chromosomes of *Pteropus dasymallus inopinatus,* a species of the Macrochiroptera, were investigated in male germ cells. This species was found to possess the diploid number of 38 in spermatogonia and the haploid number of 19 in both of the primary and secondary spermatocytes. The karyotype of this form is noticeable in having an archaic constitution in comparison with that of other forms of mammals. Excepting a pair of elongated rod-shaped elements and a very minute Y chromosome, the remaining members of the diploid complex are all provided with atelomitic fibre attachment; the majority are J- or V-shaped. The sex chromosome mechanism was shown to be of the usual XY type. The X is represented by one of the large J-shaped elements, consisting of two arms of very unequal length, while the Y is a minute dot, the smallest of all. At meiosis the Y is found conjugated with the X at the free end of its longer arm. In the first division the X and the Y pass to opposite poles. There result two kinds of secondary spermatocytes, one with an X and the other with the Y.

Observations upon the primary spermatocyte chromosomes reveal that *Rhinolophus ferrum-equinum nippon* possesses a haploid number of 29. From this haploid number it may be assumed that the diploid number of this species is 58. Judging from the configuration of the bivalents, this complement comprises both telomitic and atelomitic members. A heteromorphic XY bivalent is very distinct in the haploid group. It consists of a larger rod-shaped component, the X, and a minute dot-like one, the Y, connected end to end. They segregate in the first division.

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